NOVEL GLUCOSE TRANSPORTER/SENSOR PROTEIN AND USES THEREOF

Statement of Government Interest

This invention is supported by NIH Grant Nos. DK47425 and HL58119. As such, the U.S. Government has certain rights in this invention.

Related Application

This application is a continuation-in-part of co-pending U.S. Patent Application No. 09/356,602, filed July 19, 1999, the entire contents of which are expressly incorporated by reference herein.

Background of the Invention

Insulin stimulates the uptake of glucose from the blood into peripheral tissues, specifically skeletal muscle and adipose tissue. Glucose homeostasis is impaired in type II diabetes due, at least in part, to a defect in glucose disposal (DeFronzo, R.A., *Diabetes* 37: 667-687, 1998). More than 16 million Americans have type II diabetes, previously referred to as non-insulin-dependent diabetes mellitus (NIDDM). Type II diabetes affects nearly every organ system and may lead to a number of life-threatening conditions, including diabetic ketoacidosis, hypoglycemic coma and nonketotic hyperosmolar coma. Other complications of diabetes include retinopathy, diabetic nephropathy, diabetic neuropathy, gangrene of the feet, hypertension, atherosclerosis, and other types of coronary arterial disease.

One of the first measurable defects in type II diabetes is insulin resistance in skeletal muscle and adipose tissue. Skeletal muscle is quantitatively the most important tissue involved in maintaining glucose homeostasis, and accounts for approximately 80% of glucose disposal under insulin action (DeFronzo, et al., *J Clin Invest* 76: 149-155, 1985; DeFronzo, et al., *Diabetes* 30: 1000-1007, 1981). Glucose transport into skeletal muscle and adipose tissue is rate-limiting for glucose utilization under certain conditions, and is defective in type II diabetes (DeFronzo,

R.A., *Diabetes* 37: 667-687, 1998; Zierath, J.R., *Acta Physiol Scand Suppl* 626: 1-96, 1995; Rothman, et al., *J Clin Invest* 89: 1069-75, 1992). GLUT1 and GLUT4 are two structurally related glucose transporters expressed in insulin sensitive tissues such as adipocytes and skeletal muscle (Kahn, B.B., *Diabetes* 45: 1644-54, 1996; Devaskar, S.U. and Mueckler, M.M., *Pediatr Res* 31: 1-13, 1992). GLUT4 is the predominant facilitative glucose transporter expressed in insulin responsive tissues such as cardiac and skeletal muscle and adipose tissue (Kahn, B.B., *Diabetes* 45: 1644-54, 1996; Devaskar, S.U. and Mueckler, M.M., *Pediatr Res* 31: 1-13, 1992). Under basal conditions, GLUT4 is sequestered from the plasma membrane in a unique intracellular compartment. In response to various stimuli (i.e. insulin, contraction, hypoxia) GLUT4 translocates to the cell surface and is mostly responsible for the increase in glucose uptake (Klip, A., et al., *FEBS letters* 224: 224-230, 1987; Hirshman, et al., *J Biol Chem* 265: 987-91, 1990; Cushman, S.W. and Salans, L.B., *J Lipid Res* 19: 269-273, 1978; Douen, et al., *J Biol Chem* 265: 13427-13430, 1990; Cartee, et al., *J Appl Physiol* 70: 1593-1600, 1991). GLUT1, on the other hand, is of relatively low abundance *in vivo* in muscle and fat tissue and is primarily responsible for basal glucose uptake (Zorzano, et al., *J Biol Chem* 264: 12358-63, 1989; Holman, et al., *J Biol Chem* 265: 18172-9, 1990; Kahn, B.B., *Diabetes* 45: 1644-54, 1996; Douen, et al., *J Biol Chem* 265: 13427-13430, 1990; Gumá, et al., *Am J Physiol* 268: E613-E622, 1995). In established muscle (i.e. L6, C2/C12) and adipocyte (i.e. 3T3-L1, 3T3-F442A) cell lines, expression of GLUT1 and GLUT4 protein is atypical in that much more GLUT1 can be measured ((Zorzano, et al., *J Biol Chem* 264: 12358-63, 1989; Holman, et al., *J Biol Chem* 265: 18172-9, 1990; Calderhead, et al., *J Biol Chem* 265: 13801-8, 1990; Wilson, et al., *FEBS Lett* 368: 19-22, 1995; Garcia de Herreros, A. and Birnbaum, M.J., *J Biol Chem* 264: 19994-9, 1989). *In vivo*, particularly in skeletal muscle, GLUT1 protein is of very low abundance and most of it is localized to the perineurium (Froehner, et al., *J Neurocytol* 17: 173-178, 1988).

The insulin-sensitive glucose transporter, GLUT4, was genetically ablated in mice (Katz, et al., *Nature* 377, 151-155, 1995). Unexpectedly, GLUT4 null mice

were able to maintain normal glycemia with moderate fed hyperinsulinemia even though *in vitro* studies show the null muscle to be highly insulin resistant (Katz, et al., *Nature* 377, 151-155, 1995; Stenbit, et al., *J Clin Invest* 98, 629-634, 1996). Interestingly, oxidative soleus muscle of female GLUT4 null mice retained a significant insulin stimulated glucose uptake (Stenbit, et al., *J Clin Invest* 98, 629-634 (1996)). Furthermore, skeletal muscle of GLUT4 null mice maintained normal levels of high energy phosphate pools (Zierath, J.R. et al, *J Biol Chem* 273, 20910-20915, 1998). However, unlike serum glucose level, fed serum free fatty acids and fasted ketone body levels were significantly decreased in GLUT4 null mice (Katz, et al., *Nature* 377, 151-155, 1995). Generally, the metabolic profile of GLUT4 null mice resembled that of endurance trained athletes (reduced adiposity, cardiac hypertrophy, reduced serum fatty acids, etc.).

The present invention is based upon the discovery of a novel insulin responsive and glycemia sensitive glucose transporter/sensor/receptor, hereinafter referred to as "GLUTx", that is instrumental in the maintenance of whole body glucose homeostasis in GLUT4 null mice. Evidence suggests that GLUTx functions in a similar manner in humans, and may also be implicated in the regulation of appetite due to its suggested function as a glucose receptor and/or sensor, as evidenced by its expression in the "obesity center" of the brain. Accordingly, GLUTx provides an attractive target for the potential treatment of obesity, Type II diabetes and its various complications, as well as other diseases and conditions associated with insulin resistance, glucose regulation and homeostasis.

Summary of the Invention

Accordingly, the present invention provides an isolated nucleic acid encoding a novel protein functioning as a glycemia sensitive glucose transporter, which may also function as a glucose receptor and/or sensor, referred to herein as GLUTx. The present invention also provides vectors comprising this nucleic acid and a host cell transformed by this vector. Also provided by the present invention is a nucleic acid probe which hybridizes to nucleic acid encoding GLUTx, a mixture of nucleic acid

probes each of which hybridizes to nucleic acid encoding GLUTx and a kit comprising one or more nucleic acid probes which hybridize to nucleic acid encoding GLUTx.

The present invention also provides a method for producing recombinant GLUTx comprising growing a host cell transformed with a vector comprising nucleic acid encoding GLUTx in culture and recovering the recombinant GLUTx from the culture. The present invention further provides purified GLUTx or an analogue thereof, as well as an agent that binds to GLUTx or its analogue, including but not limited to an antibody immunoreactive with GLUTx or an analogue thereof. In addition, the present invention provides an agent which potentiates or enhances expression of GLUTx.

The present invention also provides a method for screening an agent that binds to the nucleic acid encoding a GLUTx protein comprising contacting the nucleic acid with an agent of interest and assessing the ability of the agent to bind to the nucleic acid. The present invention further provides for a method for screening an agent that inhibits or enhances the expression of the nucleic acid encoding GLUTx protein comprising contacting a cell transformed with a vector comprising the nucleic acid, and assessing the effect of the agent on expression of the nucleic acid. The present invention still further provides a method for screening for an agent that binds to a GLUTx protein or an analogue thereof comprising contacting the GLUTx protein with an agent of interest and assessing the ability of the agent to bind to GLUTx.

The present invention also provides a method for treating conditions which are related to insufficient activity of GLUTx, including but not limited to type-II diabetes and/or obesity, comprising administering to a subject a nucleic acid molecule comprising a nucleotide sequence encoding GLUTx, such that the administered nucleic acid sequence is expressed in target cells of the patient, thereby alleviating the treated condition. In addition, the present invention provides methods for treating diseases or conditions characterized by insufficient activity of GLUTx, including but not limited to type-II diabetes and/or obesity, by administering

an agent which potentiates or enhances expression of GLUTx.

Further provided is a recombinant viral vector capable of introducing nucleic acid encoding GLUTx into a target cell such that the target cell expresses the GLUTx protein, the vector comprising nucleic acid of or corresponding to at least a portion of the genome of a virus, the portion being capable of infecting the target cell, and nucleic acid encoding a GLUTx protein operably linked to the viral nucleic acid. Finally, the present invention provides a non-human, transgenic animal model comprising nucleic acid encoding GLUTx, or a mutated nucleic acid encoding GLUTx, incorporated into at least some of the somatic cells of the animal, as well as a method for the use of such transgenic animals as models for disease states. Additional objects of the present invention will be apparent from the description which follows.

Brief Description of the Figures

Figure 1 depicts Northern analysis of GLUTx clone. Total RNA from different muscles and brain was extracted from control (C) and GLUT4 null (N) animals. 50 micrograms of total RNA was loaded into each lane. High stringency hybridization to a random prime labeled GLUTx probe is shown in the upper panel. The filter was also hybridized with tubulin to assess equality of loading.

Figure 2 depicts the upregulation of GLUTx mRNA expression in fat (white (WAT) and brown (BAT) adipose tissue), heart and diaphragm.

Figure 3 illustrates *in vivo* glucose utilization under clamp conditions. (A) Whole body glucose utilization rates of control and GLUT4 null mice under basal (6 hours fasted), euglycemic (100mg/dl) hyperinsulinemic and mildly hyperglycemic (200mg/dl) hyperinsulinemic clamp conditions. Asterisks (*) indicate $p<0.05$ between control and GLUT4 null groups using Student's *t*-test. n=6-8 in each group. (B) 2-Deoxyglucose uptake in individual tissues under euglycemic hyperinsulinemic (left panel) and mildly hyperglycemic hyperinsulinemic (right panel) clamp conditions. n=6-8 in each group. BAT: brown adipose tissue, Diaph: diaphragm, EDL: extensor digitorum longus. Asterisks (*) indicate $p<0.05$ between control and

GLUT4 null groups using Student's *t*-test. (C) Glycogen accumulation at the end of basal (hatched bars), euglycemic hyperinsulinemic (open bars) and mildly hyperglycemic hyperinsulinemic (solid bars) clamp studies. Asterisks (*) indicate $p<0.05$ by ANOVA. n=6-8 in each group.

Figure 4. (A) Glycogen accumulation in isolated soleus and EDL muscle of control and GLUT4 null mice under basal (open bars) and insulin stimulated (solid bars) conditions. n=8-10 in each group. (B) Percentage of glucose oxidation, glycolysis and glycogen synthesis of total glucose utilization in isolated soleus and EDL muscle of GLUT4 null and control mice under basal and insulin stimulated conditions. (C) Oral olive oil tolerance test of GLUT4 null and control mice. Open circle: GLUT4 null, filled circle, control. n=6 in each group. (D) Oleate oxidation rates in isolated soleus and EDL muscles of control and GLUT4 null mice. In all panels, asterisks (*) denote $p<0.05$ between control and GLUT4 null groups using Student's *t*-tests. n=8-10 in each group.

Figure 5. (A) Transmission electron micrographs of mitochondria from soleus and EDL of GLUT4 null and control mice. Representative micrographs are shown at 10,000X magnification. (B) Representative immunoblot analysis of mitochondrial outer membrane proteins in soleus of GLUT4 null and control mice. Results from 6 samples in each group were quantitated by densitometry. (C) Northern blot analysis of mitochondrial inorganic phosphate (Pi) carrier and COX IV gene expression in GLUT4 null and control hindlimb muscle. RNA loading was normalized using 18S rRNA. Results from 6 samples in each group were quantitated by densitometry. (D) Northern blot analysis of glucokinase (GK) and fatty acid synthase (FAS) gene expression in GLUT4 null and control liver. Results from 6 samples in each group were normalized and quantitated as in (C).

Figure 6 illustrates sequence alignment between murine GLUT4 and GLUTx. Shaded regions indicate sequence similarity. Boxed regions indicate sequence identity.

Figure 7 illustrates sequence alignment between *Saccharomyces* RGT2, SNF3 and GLUTx. Shaded regions indicate sequence similarity. Boxed regions indicate

sequence identity.

Figure 8 depicts Northern Blot analysis of GLUTx, identifying an approximately 2.4 kb major RNA in tissues such as skeletal muscle, diaphragm, heart, fat, spleen, brain and liver.

Figure 9 depicts a partial nucleotide sequence of Human GLUTx. The putative stop is depicted as T G A.

Figure 10 depicts the predicted amino acid sequence encoded by the nucleotide sequence of Figure 9. "*" indicates the putative carboxy terminus of the GLUTx protein.

Figure 11 depicts a partial nucleotide sequence of rat GLUTx.

Figure 12 depicts the predicted amino acid sequence encoded by the nucleotide sequence of Figure 11.

Figure 13 depicts a partial nucleotide sequence of mouse GLUTx.

Figure 14 depicts the predicted amino acid sequence encoded by the nucleotide sequence of Figure 13.

Figure 15 illustrates 3-O-methylglucose transport under basal and insulin-stimulated conditions. Muscles from control (open bars) and GLUT4-null (closed bars) mice were pre-incubated in presence or absence of 20nM insulin for 30 min, followed by incubation for 15 (basal) or 7 min (insulin) in KHB containing 5 mM 3-O-methylglucose. Values are mean ±SEM for n=5-11 muscles per group. *$p<0.01$ vs. basal. $p<0.001$ vs. control mice.

Figure 16 depicts *in vitro* glycolysis rates in isolated soleus and EDL muscles from female control and GLUT4 null mice under basal (shaded bars) and insulin stimulated (open bars) conditions. $p<0.05$ between GLUT4 null and controls. Results shown are mean ±SEM of 7-12 muscles/group.

Figure 17 illustrates *in vitro* glycogen synthesis in isolated soleus and EDL muscles from female control and GLUT4 null mice under basal (shaded bars) and insulin stimulated (open bars) conditions. $p<0.05$ between GLUT4 null and controls. Results are mean ±SEM of 7-12 muscles/group.

Figure 18 depicts *in vitro* glucose oxidation rates in isolated soleus and EDL

muscles from female control and GLUT4 null mice under basal (shaded bars) and insulin stimulated (open bars) conditions. $p<0.05$ between GLUT4 null and controls. Results are mean ±SEM of 7-12 muscles/group.

Detailed Description of the Invention

The present invention provides a purified and isolated nucleic acid encoding GLUTx. As used herein, the nucleic acid may be genomic DNA, cDNA, RNA, as well as antisense analogs thereof and diagnostically or therapeutically useful fragments thereof, and includes nucleic acid derived from any species, e.g., human, rat, goat, pig, mouse and cow, although most preferably is derived from human. Due to the degeneracy of the genetic code, the nucleic acid of the present invention also includes a multitude of nucleic acid substitutions which will encode GLUTx. The nucleic acid from the human preferably encodes for the amino acid sequence for human GLUTx as shown in Figure 10 (preferably comprising the amino acid sequence from the beginning of Figure 10 to the putative carboxy terminus as depicted in Figure 10), and more preferably comprises the nucleotide sequence as shown in Figure 9 (and preferably comprising the 1362 bp nucleotide sequence from CGC to TGA, as depicted in Figure 9). The nucleic acid from the rat preferably encodes the amino acid sequence for rat GLUTx shown in Figure 12, and more preferably comprises the nucleotide sequence shown in Figure 11. The nucleic acid from the mouse preferably encodes for the amino acid sequence for mouse GLUTx as shown in Figure 14, and more preferably comprises the nucleotide sequence shown in Figure 13. The present invention also includes nucleic acid sequences encoding proteins having GLUTx activity that are at least 80%, preferably at least 85%, more preferably at least 90%, even more preferably at least 95%, and most preferably, 98% homologous with any of the nucleic acid sequences set forth above.

Also provided by the present invention is an isolated and purified nucleic acid that hybridizes under high stringency conditions (*i.e.*, hybridization to filter bound DNA in 0.5M $NaHPO_4$ at 65°C and washing in 0.1X SSC/0.1% SDS at 68°C) or moderate stringency conditions (*i.e.*, washing in 0.2X SSC/0.1% SDS at 42°C)

(Ausubel, F.M. *et al.*, 1998 Current Protocols in Molecular Biology) to the nucleic acid sequence of Figure 9 (or, preferably, to the 1362 bp nucleotide sequence from CGC to TGA, as depicted in Figure 9), or to the complement of the nucleic acid sequence of Figure 9 (or, preferably, to the complement of the 1362 bp nucleotide sequence from CGC to TGA, as depicted in Figure 9), wherein said isolated nucleic acid encodes a protein having GLUTx biological activity. Further provided is an isolated and purified nucleic acid that hybridizes under high stringency conditions or moderate stringency conditions to the nucleic acid sequence of Figure 11, or to the complement of the nucleic acid sequence of Figure 11, wherein said isolated nucleic acid encodes a protein having GLUTx biological activity. Still further provided is an isolated and purified nucleic acid that hybridizes under high stringency conditions or moderate stringency conditions to the nucleic acid sequence of Figure 13, or to the complement of the nucleic acid sequence of Figure 13, wherein said isolated nucleic acid encodes a protein having GLUTx biological activity.

In addition, the present invention provides the nucleic acid encoding the GLUTx protein having one or more mutations resulting in the expression of a non-functional or mutant protein, or in lack of expression altogether. The mutation may be one or more point, insertion, rearrangement or deletion mutations or a combination thereof.

The present invention also provides a vector which comprises nucleic acid encoding GLUTx. Such vectors may be constructed by inserting nucleic acid encoding GLUTx into suitable vector nucleic acid. The term "inserted" as used herein means the ligation of a foreign DNA fragment and vector DNA by techniques such as the annealing of compatible cohesive ends generated by restriction endonuclease digestion or by use of blunt end ligation techniques. Other methods of ligating DNA molecules will be apparent to one skilled in the art. Vectors may be derived from a number of different sources. They can be plasmids, viral-derived nucleic acids, lytic bacteriophage derived from phage lambda, cosmids or filamentous single-stranded bacteriophages such as M13. Depending upon the type of host cell into which the vector is introduced, vectors may be bacterial or eukaryotic. Bacterial vectors are

derived from many sources including the genomes of plasmids and phages. Eukaryotic vectors are also constructed from a number of different sources, e.g. yeast plasmids and viruses. Some vectors, called shuttle vectors, are capable of replicating in both bacteria and eukaryotes. The nucleic acid from which the vector is derived is usually greatly reduced in size so that only those genes essential for its autonomous replication remain. The reduction in size enables the vectors to accommodate large segments of foreign DNA. Examples of suitable vectors into which the nucleic acid encoding GLUTx can be inserted include but are not limited to pBR322, pUC18, pUC19, pACYC177, pKT230, pHV14, pHSV-106, pJS97, pJS98, M13mp18, M13mp19, pSPORT 1, pGem, pSPORT 2, pSV•SPORT 1, pBluescript II, pIJ61, pUC6, λZapII, λgt10, λgt11, λgt22A, and λZIPLOX. Other suitable vectors are obvious to one skilled in the art.

The vector of the present invention may be introduced into a host cell and may exist in integrated or unintegrated form within the host cell. When in unintegrated form, the vector is capable of autonomous replication. The term "host cell" as used herein means the bacterial or eukaryotic cell into which the vector is introduced. As used herein, "introduced" is a general term indicating that one of a variety of means has been used to allow the vector to enter the intracellular environment of the host cell in such a way that it exists in stable and expressable form therein.

Some bacterial and eukaryotic vectors have been engineered so that they are capable of expressing inserted nucleic acids to high levels within the host cell. Such vectors utilize one of a number of powerful promoters to direct the high level of expression. For example, in vectors for the expression of a gene in a bacterial host cell such as E.coli, the lac operator-promoter or the tac promoter are often used. Eukaryotic vectors use promoter-enhancer sequences of viral genes, especially those of tumor viruses. Expression can be controlled in both bacterial and eukaryotic cells using inducible promoters such as the lac operator-promoter in E.coli or metallothionine or mouse mammary tumor virus promoters in eukaryotic cells. As used herein, "expression" refers to the ability of the vector to transcribe the inserted

nucleic acid into mRNA so that synthesis of the protein encoded by the inserted nucleic acid can occur.

Vectors suitable for the expression of the nucleic acid encoding GLUTx in a host cell are well known to one skilled in the art and include pET-3d (Novagen), pProEx-1 (Life Technologies), pFastBac 1 (Life Technologies), pSFV (Life Technologies), pcDNA II (Invitrogen), pSL301 (Invitrogen), pSE280 (Invitrogen), pSE380 (Invitrogen), pSE420 (Invitrogen), pTrcHis A,B,C (Invitrogen), pRSET A,B,C (Invitrogen), pYES2 (Invitrogen), pAC360 (Invitrogen), pVL1392 and pVl1392 (Invitrogen), pCDM8 (Invitrogen), pcDNA I (Invitrogen), pcDNA I(amp) (Invitrogen), pZeoSV (Invitrogen), pcDNA 3 (Invitrogen), pRc/CMV (Invitrogen), pRc/RSV (Invitrogen), pREP4 (Invitrogen), pREP7 (Invitrogen), pREP8 (Invitrogen), pREP9 (Invitrogen), pREP10 (Invitrogen), pCEP4 (Invitrogen), pEBVHis (Invitrogen), and λPop6. Other vectors would be apparent to one skilled in the art.

Vectors may be introduced into host cells by a number of techniques known to those skilled in the art, e.g. electroporation, DEAE dextran, cationic liposome fusion, protoplast fusion, DNA coated-microprojectile bombardment, and infection with recombinant replication-defective retroviruses. The term "transformation" denotes the introduction of a vector into a bacterial or eukaryotic host cell. As such, it encompasses transformation of bacterial cells and transfection, transduction and related methods in eukaryotic cells.

Any one of a number of suitable bacterial or eukaryotic host cells may be transformed with the vector of the present invention. Examples of suitable host cells are known to one skilled in the art and include but are not limited to bacterial cells such as E.coli strains c600, c600hfl, HB101, LE392, Y1090, JM103, JM109, JM101, JM107, Y1088, Y1089, Y1090, Y1090(ZZ), DM1, PH10B, DH11S, DH125, RR1, TB1 and SURE, Bacillus subtilis, Agrobacterium tumefaciens, Bacillus megaterium; and eukaryotic cells such as Pichia pastoris, Chlamydomonas reinhardtii, Cryptococcus neoformans, Neurospora crassa, Podospora anserina, Saccharomyces cerevisiae, Saccharomyces pombe, Uncinula necator, cultured insect cells, cultured chicken fibroblasts, cultured hamster cells, cultured human cells such as HT1080, MCF7,

143B and cultured mouse cells such as EL4 and NIH3T3 cells.

The present invention also provides a method for producing a recombinant GLUTx protein comprising growing a host cell transformed with a vector encoding GLUTx in culture and recovering recombinant GLUTx. As used herein the term "recombinant" refers to GLUTx produced by purification from a host cell transformed with a vector capable of directing its expression to a high level. A variety of methods of growing host cells transformed with a vector are known to those skilled in the art. The type of host cell, i.e. whether the host cell is bacterial or eukaryote, is the primary determinant of the method to be utilized and the optimization of specific parameters relating to such factors as temperature, trace nutrients, humidity, and growth time. Depending on the vector, the host cells may have to be induced by the addition of a specific compound at a certain point in their growth cycle in order to initiate expression of the nucleic acid of the present invention. Examples of compounds generally used to induce expression of nucleic acids are known to one skilled in the art and include but are not limited to IPTG, zinc and dexamethasone. Using standard methods of protein isolation and purification, such as ammonium sulfate precipitation followed by dialysis to remove salt, followed by fractionation according to size, charge of the protein at specific pH values, affinity methods, etc., recombinant GLUTx may be extracted from suitable host cells transformed with vector capable of expressing the nucleic acid encoding GLUTx.

The present invention also provides purified GLUTx protein and analogues thereof and includes GLUTx isolated from tissue obtained from a subject or recombinantly produced as described above. As used herein an "analogue" may be any protein having GLUTx activity and is at least 85%, more preferably at least 90%, even more preferably at least 95%, and most preferably, 98% homologous to the any of the amino acid sequences of Figures 10 (including an amino acid sequence ending at the putative carboxy terminus), 12 or 14. In a preferred embodiment, the purified GLUTx protein of the present invention has a molecular weight of approximately 32.6 kD and is encoded by the amino acid sequence for human GLUTx as shown in Figure 10 (including an amino acid sequence ending at the

putative carboxy terminus as depicted in Figure 10), and more preferably is encoded by the corresponding nucleic acid sequence for human GLUTx as shown in Figure 9 or by a nucleic acid sequence that hybridizes to the complement of the corresponding nucleic acid sequence as shown in Figure 9 under moderate or, preferably, high stringency conditions. In an alternate embodiment of the invention, the purified GLUTx has a molecular weight of approximately 32.6 kD and is encoded by the amino acid sequence for rat GLUTx as shown in Figure 12, and more preferably is encoded by the nucleic acid sequence for rat GLUTx as shown in Figure 11 or by a nucleic acid sequence that hybridizes to the complement of the nucleic acid sequence as shown in Figure 11 under moderate or, preferably, high stringency conditions. In yet another alternate embodiment, the GLUTx protein of the present invention has a molecular weight of approximately 32.6 kD, and is encoded by the amino acid sequence for mouse GLUTx as shown in Figure 14, and more preferably is encoded by the nucleic acid sequence for mouse GLUTx as shown in Figure 13 or by a nucleic acid sequence that hybridizes to the complement of the nucleic acid sequence as shown in Figure 13 under moderate or, preferably, high stringency conditions..

The present invention also includes a mutated GLUTx, i.e., GLUTx which is inactive or only has minimal effects *in vivo*. The non-functional GLUTx protein may have one or more deletions, insertions or substitutions of its amino acid or encoding nucleotide sequence that results in the GLUTx protein losing its functionality. Mutants may be prepared by any number of methods known to those skilled in the art, including site-directed mutagenesis, inverse PCR reactions, etc.

The present invention also provides for agents that bind to GLUTx and analogues thereof, as well as the non-functional GLUTx protein. The agent may be a antibody, a nucleic acid, a protein, a peptide, DNA, RNA, mRNA, antisense RNA, a drug or a compound. Agents that bind to GLUTx or an analogue thereof may be identified or screened by contacting the protein with the agent of interest and assessing the ability of the agent to bind to the protein. Agents that bind to the GLUTx protein may act as agonists and thereby change the configuration of GLUTx,

alone or in association with other agents, to increase sensitivity of the GLUTx protein to glucose, or may increase glucose transport activity of GLUTx. Such agonists may be useful as therapeutic agents for type-II diabetes and/or obesity, as well as complications from these conditions. Agents that bind to GLUTx may also act as antagonists, thereby reducing the activity of GLUTx. Such agents may be useful in creating disease models and the like.

Antibodies immunoreactive with GLUTx or analogues thereof include antibodies immunoreactive with non-functional GLUTx protein. The antibodies of the present invention may be monoclonal or polyclonal and are produced by techniques well known to those skilled in the art, e.g., polyclonal antibody can be produced by immunizing a rabbit, mouse, or rat with purified GLUTx and monoclonal antibody may be produced by removing the spleen from the immunized rabbit, mouse or rat and fusing the spleen cells with myeloma cells to form a hybridoma which, when grown in culture, will produce a monoclonal antibody. Labeling of the antibodies of the present invention may be accomplished by standard techniques using one of the variety of different chemiluminescent and radioactive labels known in the art. The antibodies of the present invention may also be incorporated into kits which include an appropriate labeling system, buffers and other necessary reagents for use in a variety of detection and diagnostic applications.

The present invention provides for agents that bind to a nucleic acid encoding GLUTx protein. Suitable agents include but are not limited to a nucleic acid, a protein, a peptide, DNA, RNA, mRNA, antisense nucleic acids, a drug or a compound. Preferably, the agents enhance expression of the GLUTx nucleic acid, for instance, by functioning as a transcription factor, an activator, or a repressor binding to silencer sites preventing activator binding or transcription. Agents binding to enhancer sites upregulating GLUTx are also covered by the present invention.

Such agents as noted above may be discovered by a method for screening for an agent that binds to the nucleic acid of GLUTx comprising contacting the nucleic acid with an agent of interest and assessing the ability of the agent to bind to the nucleic acid. An agent that regulates the expression of the nucleic acid encoding the

GLUTx protein may be screened by contacting a cell transformed with a vector comprising the nucleic acid, and assessing the effect of the agent on expression of the nucleic acid. Agents that bind to the nucleic acid encoding GLUTx may act to upregulate expression of GLUTx and thereby promote glucose homeostasis or potentially the control of appetite in a subject.

The present invention also provides nucleic acid probes and mixtures thereof which are hybridizable to the nucleic acid encoding GLUTx. Such probes may be prepared by a variety of techniques known to those skilled in the art such as PCR and restriction enzyme digestion of GLUTx nucleic acid or by automated synthesis of oligonucleotides whose sequence correspond to selected portions of the nucleotide sequence of the GLUTx nucleic acid using commercially available oligonucleotide synthesizers such as the Applied Biosystems Model 392 DNA/RNA synthesizer. The nucleic acid probes of the present invention may also be prepared so that they contain one or more point, insertion, rearrangement or deletion mutations or a combination thereof to correspond to mutations of the GLUTx gene. The nucleic acid probes of the present invention may be DNA or RNA and may vary in length from about 8 nucleotides to the entire length of the GLUTx nucleic acid. Preferably, the probes are 8 to 30 nucleotides in length. Labeling of the nucleic acid probes may be accomplished using one of a number of methods known in the art, e.g., PCR, nick translation, end labeling, fill-in end labeling, polynucleotide kinase exchange reaction, random priming, or SP6 polymerase (for riboprobe preparation) and one of a variety of labels, e.g., radioactive labels such as ^{35}S, ^{32}P or ^{3}H or nonradioactive labels such as biotin, fluorescein (FITC), acridine, cholesterol or carboxy-X-rhodamine (ROX). Combinations of two or more nucleic probes corresponding to different or overlapping regions of the GLUTx nucleic acid may also be included in kits for use in a variety of detection and diagnostic applications.

The present invention also provides a method for detecting abnormal expression of GLUTx which, if overexpressed, may be associated with reduced adiposity, cardiac hypertrophy, reduced serum and free fatty acids, etc. Increased GLUTx expression may also be indicative of impaired GLUT4 function or an elevated

physiological stress level. Increased or decreased expression of GLUTx may be determined by nucleic acid hybridization and/or immunological techniques well known in the art. For example, nucleic acid hybridization using mRNA extracted from cells and GLUTx nucleic acid probes can be used to determine the concentration of GLUTx mRNA present in the cell and the concentration thus obtained compared to the value obtained for cells which exhibit a normal level of GLUTx activity. Isolation of RNA from cells is well known in the art and may be accomplished by a number of techniques, e.g., whole cell RNA can be extracted using guanidine thiocyanate; cytoplasmic RNA may be prepared by using phenol extraction methods; and polyadenylated RNA may be selected using oligo-dT cellulose. Alternatively, the concentration of GLUTx in the cell may be determined from binding studies using antibody immunoreactive with GLUTx.

The method of the present invention also provides a non-human animal model for the study of GLUTx expression. The animal model of the present invention comprises a non-human, transgenic animal having nucleic acid encoding a GLUTx protein incorporated into at least some of the somatic cells of the animal. The effect of the expression of the GLUTx protein also may be studied by overexpressing or underexpressing the protein using suitable promoters and regulators known in the art. It is also within the confines of the present invention that a nucleic acid sequence having one or more mutations may be introduced into the animal model that result in the expression of a non-functional or mutant protein. Nucleic acid encoding mutated GLUTx may be integrated into the germ line of a non-human animal such as a mouse, rat, goat, sheep or other species in order to obtain a transgenic animal. Expression of the incorporated nucleic acid may be restricted to certain tissues in the transgenic animal by the utilization of tissue-specific promoters. Methods of making transgenic animals are well known in the art. For example, DNA encoding mutated GLUTx can be inserted into the genome of a replication-defective virus such as HSV, or a retrovirus or transposon and the resultant construct injected into embryonic stem cells. Transgenic animals may also be made by injecting DNA encoding mutated GLUTx into the male pronucleus of a

fertilized egg of a nonhuman animal, transplanting the "transgenic embryo" into a pseudopregnant female and then analyzing offspring for the presence of the injected DNA in their genome. Other methods of producing transgenic mice would be apparent to one skilled in the art.

The present invention provides a method of treating type-II diabetes, obesity or other diseases and conditions resulting from insulin resistance, underexpression of GLUTx or expression of nonfunctional GLUTx, comprising administering to a subject a nucleic acid molecule comprising a nucleotide sequence encoding the amino acid sequence of Figure 10 (preferably a nucleotide sequence comprising the nucleotide sequence of Figure 9), such that said nucleic acid sequence is expressed in target cells of the patient thereby alleviating the type-II diabetes or subject condition. Depending upon the condition being treated, target cells may include, but are not limited to, skeletal muscle cells, nerve cells and/or glial cells. Gene therapy of the present invention may involve the isolation and purification of the subject's cells, introduction of the GLUTx gene, and reintroduction of the altered cells back into the subject. A replication-deficient virus such as a modified retrovirus can be used to introduce the GLUTx gene. Other examples of replication-deficient viruses suitable for the introduction of the therapeutic gene *in vitro* are well known in the art.

Alternatively, nucleic acid encoding GLUTx may be administered to a patient *in vivo*, by packaging said nucleic acid in liposomes, in a replication-deficient virus such as an adenovirus or adeno-associated virus vectors, or by the direct injection of plasmid DNA or recombinant vectors, naked DNA or by other nucleic acid delivery methods as known in the art.

Finally, the present invention provides a method of treating type-II diabetes, obesity or other diseases/conditions resulting from insulin resistance, the underexpression of GLUTx or expression of nonfunctional GLUTx, in a subject, comprising administering to a subject an agent that binds to GLUTx, preferably acting as an agonist of GLUTx, or alternatively, an agent that enhances the expression of a nucleic acid encoding GLUTx, together with a physiologically acceptable carrier in an amount effective to the subject condition in the patient.

The present invention is described in the following Experimental Details Section which is set forth to aid in the understanding of the invention, and should not be construed to limit in any way the invention as defined in the claims which follow thereafter.

Experimental Details

Introduction

Gene targeting was used with embryonic stem cells to ablate GLUT4 protein in order to determine the role it plays in whole body glucose homeostasis (Katz, et al., Nature 377:151-155, 1995). Surprisingly, GLUT4 null mice are not diabetic. In fact, they display normal glucose tolerance and normal fasting and fed glucose levels. However, alterations in normal glucose and lipid metabolism do occur in GLUT4 null mice. In the fed state, there is a significant reduction in serum lactate and free fatty acids, while in the fasted state, ketones are severely reduced. In addition to having altered serum metabolites which resemble that of an endurance trained athlete, GLUT4 null mice also display severely diminished adipose tissue depots and exhibit extreme cardiac hypertrophy without hypertension (Katz, et al., Nature 377:151-155, 1995).

Surprisingly, slow twitch soleus muscle of GLUT4 null females displayed a significant increase in glucose uptake following *in vitro* incubation with insulin (Stenbit, et al., J Clin Invest 98:629-634, 1996). This result was not detected in fast twitch EDL muscle. Complementation of GLUT4 null mice with a fast-twitch specific GLUT4 transgene proved the glucose transport activity measured in GLUT4 null soleus was not dependent upon hyperinsulinemia, as the MLC-GLUT4 null mice had normal fed glucose and insulin and normal insulin tolerance (Tsao, et al., J Clin Invest 100: 671-677, 1997). *In vitro* exposure to hypoxia, another stimulus of muscle glucose uptake, failed to elicit a response in soleus and EDL of GLUT4 null mice (Zierath, et al., J. Biol. Chem. 273: 20910-20915, 1998). This result demonstrated GLUT4 was essential for the hypoxia-stimulated increase in muscle glucose uptake. To determine whether the insulin stimulated glucose uptake

measured in soleus from female GLUT4 null mice was specific, glucose uptake was measured in the presence of several known inhibitors of facilitated glucose transporters (i.e. cytochalasin B, ATB-BMPA). In each case, glucose uptake was inhibited suggesting that glucose uptake in GLUT4 null soleus is due to a transporter mediated process and not simple diffusion.

To ascertain whether the insulin stimulated glucose uptake measured in female GLUT4 null soleus was due to altered GLUT1 trafficking, cell surface photolabeling using ATB-BMPA was employed. Results of this analysis demonstrated that GLUT1 trafficking and content was unaltered in GLUT4 null soleus under basal and insulin stimulated conditions. These results strongly suggest that GLUT4 null mice maintain glucose transport into muscle cells at a level sufficient for normal function through a membrane protein (GLUTx) that is not GLUT1.

Whole body glucose disposal and carbohydrate metabolism in GLUT4 null mice was measured under basal, euglycemic/hyperinsulinemic and hyperglycemic/ hyperinsulinemic clamp conditions. While GLUT4 null mice display significant reductions in whole body glucose turnover under euglycemic and hyperglycemic clamp conditions, basal glucose turnover rates were normal. This result is not so astonishing as GLUT4 plays a most important role under insulin action. By injecting a trace amount of labeled 2-deoxyglucose (2-DOG), tissue uptake was determined. Euglycemic clamp data verified that indeed soleus muscle of GLUT4 null mice could take up a significant, though reduced, amount of glucose. Importantly, the *in vivo* euglycemic clamp study identified highly oxidative tissues which normally express GLUT4 that appear to express a glucose transport activity similar to that of GLUT4 null soleus muscle. These tissues include heart, diaphragm and brown adipose tissue (BAT). Under hyperglycemic clamp conditions, this glucose transport activity (i.e. GLUTx) expressed in GLUT4 null tissues was even more active as evidenced by further increases in glucose uptake.

Several strategies were employed to identify a novel cDNA that might be responsible for the insulin stimulated glucose uptake identified in female soleus of GLUT4 null mice. From the inhibitor studies noted above, it was reasoned that the

putative GLUTx protein retained binding sites for cytochalasin B, forskolin and ATB-BMPA. Oligonucleotide primers were designed that would encompass such sites and also contained highly conserved regions of known mammalian facilitative glucose transporters. Reverse transcriptase-PCR was used to amplify a sequence from oxidative (red) skeletal muscle of GLUT4 null mice that may encode GLUTx. The predicted amino acid sequence of the GLUTx clone has features in common with known facilitative glucose transporters (i.e. GLUT-4). The GLUTx clone also contains a sequence motif found only in glucose sensors/glucose receptors (i.e. SNF3, RGT2) of *Saccharomyces*. As noted above, GLUTx activity is enhanced under hyperglycemic conditions which suggest it might be a glucose sensor.

Northern blot analysis indicates that GLUTx mRNA (2.7 kb) is upregulated in GLUT4 null skeletal muscle, heart, and diaphragm, with the largest increase noted in diaphragm muscle (see Fig. 1). This observation is consistent with the results of *in vivo* euglycemic and hyperglycemic clamps that predicted GLUTx expression in skeletal muscle, heart and diaphragm. The insulin sensitizing drug, thiazoladinedione (TZD), regulates the expression of GLUTx mRNA in normal mice. TZD downregulates GLUTx mRNA expression in hindlimb, which it upregulates GLUTx mRNA expression in fat, heart, and diaphragm (see Fig. 2). The GLUT4 null mutation is coincident with severe cardiac hypertrophy in the absence of hypertension which is similar to that noted in athletes. Stress induced by ischemia was used to demonstrate how well adapted GLUT4 null hearts which overexpress GLUTx have become to utilizing glucose in contrast with normal hearts. After an ischemic episode, Langendorff perfused GLUT4 null hearts maintained function while normal hearts lost function. The inventors believe GLUTx represents a novel therapeutic target in the management of diabetes, ischemia, obesity and other diseases of insulin resistance.

Example 1

Methods:

(i) *In vivo* glucose utilization: Clamp studies were carried out as described previously (Kamohara, et al., *Nature* 389, 374-377, 1997; Massillon, D. *et al., Am J*

Physiol 269, E1037-E1043, 1995). Briefly, 6-9 month old female GLUT4 null and control mice were catheterized via the left femoral vein. Mice were allowed to recover for 3 days after surgery. Food was removed 5-6 hours before the infusion. After a bolus infusion (5 μCi), 3-^{3}H-glucose was infused at the rate of 7μCi/min/kg to assess whole body glucose utilization rate (Rd). During hyperinsulinemic clamps, insulin was infused at 18 mU/min/kg for clamps performed at 100 mg/dl and at 100 mU/min/kg for 200 mg/dl clamp studies. The increase in insulin infusion rate had no effect on Rd in both control and GLUT4 null mice. Exogenous glucose was infused at variable rates to reach the desired glucose concentrations. Blood glucose levels were monitored by tail vein sampling. Steady state was reached approximately 100 minutes after starting the infusion, at which point 20 μCi of ^{14}C-2-deoxyglucose was flash injected through the catheter 30 min later to assess individual tissue glucose uptake rates. 40 minutes after the 2-DOG injection, mice were sacrificed and tissues were quickly removed for further analysis. Whole body glucose utilization and tissue 2-DOG uptake was calculated as described previously (Kamohara, et al., *Nature* 389, 374-377, 1997; Massillon, D. *et al., Am J Physiol* 269, E1037-E1043, 1995). Tissue glycogen was analyzed as described previously (Tsao, T.S., et al., *Diabetes* 45, 28-36, 1996; Chan, T.M. et al., *Anal Biochem* 71, 96-105, 1976).

(ii) Oral olive oil loading test: Oral olive oil loading test was conducted as described previously (Abe, H. *et al., J Clin Invest* 101, 1784-1788, 1998). Briefly, mice were fasted for 5 hours. Olive oil was delivered by a gavage needle at 16.7 μl/gram body weight. Blood samples were obtained immediately before and 30, 60, 120 180, and 300 mins after oil loading. Serum triglyceride levels were analyzed spectrophotometrically using a kit from Sigma, Inc.

(iii) *In vitro* glucose and oleate utilization: Soleus and EDL muscle of 10-14 week old female GLUT4 null and age-/sex-matched control mice were isolated as described previously (Stenbit, A.E. *et al.* , *J Clin Invest* 98, 629-634, 1996). To determine glucose oxidation, glycolysis and glycogen synthesis rates, muscles were incubated in Krebs-Ringer buffer containing 0.1 mCi/ml U-^{14}C-glucose and 1 mCi/ml

5-^{3}H-glucose with or without 20 nM insulin. Vials were capped with a screw top lid containing a rubber septum from which a center well containing a 2×3 cm piece of Whatman paper was suspended. Each vial was aerated for 45 minutes and incubated for an additional hour under closed conditions. Released $^{14}CO_2$ was captured on the Whatmann paper by injecting Solvable (Amersham, Inc.) to the paper and perchloric acid into the medium. $^{14}CO_2$ counts were used to calculate glucose oxidation rate. Glycolysis rate was calculated by the accumulation of $^{3}H_2O$ in the incubation medium (Cuendet, G.S., et al., *J Clin Invest* 58, 1078-1088, 1976). Glycogen synthesis rates were determined by the accumulation of either ^{3}H or ^{14}C in glycogen purified from individual muscles (Chan, T.M. et al., *Anal Biochem* 71, 96-105, 1976; Karl, I.E., et al., *Diabetes* 39, 1106-1115, 1990).

(iv) To determine oleate oxidation rates, isolated muscles were incubated in Krebs-Ringer buffer containing 5mM glucose, 0.5 mM oleate and 0.8 μCi/ml 1-^{14}C-oleate. The $^{14}CO_2$ formed from 1-^{14}C-oleate oxidation was captured as described in the glucose oxidation study and used to assess oleate oxidation rates.

(v) Immunoblot analysis: Immunoblot analysis was performed as described previously (Stenbit, A.E. *et al.* , *J Clin Invest* 98, 629-634, 1996). A rabbit polyclonal antibody (1:1000) against purified mitochondrial outer membrane proteins (obtained from Dr. P. E. Scherer, Albert Einstein College of Medicine) was used to detect expression of mitochondrial outer membrane proteins in total homogenates (100 μg) of soleus and EDL. Samples were prepared as described (Scherer, P.E. *et al., J Cell Biol* 127, 1233-1243, 1994).

(vi) Northern blot analysis: Northern blot analysis was performed as described previously (Li, J., et al., *BBA* 1356, 229-236, 1997). cDNA fragments of murine COX IV, inorganic phosphate carrier (Pi), glucokinase (GK) and fatty acid synthase (FAS) were used as templates for synthesizing random-primed ^{32}P labeled probes. Loading was normalized by 18S rRNA. Results from 6 animals in each group were quantified by densitometry.

(vii) Statistical analysis: Data are presented as mean±SE of multiple

determinations. Statistical significance was evaluated by two-tailed, unpaired, Student's t-test or by ANOVA using Fisher's PLSD for post hoc analysis. Significance was accepted at $p<0.05$.

Results:

The maintenance of post-prandial glucose homeostasis requires insulin-stimulated glucose transport into skeletal muscle (Kahn, B.B., *Diabetes* 45, 1644-1654, 1996). The insulin-sensitive glucose transporter, GLUT4, was genetically ablated in mice (Katz, et al., *Nature* 377, 151-155, 1995). Unexpectedly, GLUT4 null mice are able to maintain normal glycemia with moderate fed hyperinsulinemia even though *in vitro* studies show the null muscle to be highly insulin resistant (Katz, et al., *Nature* 377, 151-155, 1995; Stenbit, A.E. et al, *J Clin Invest* 98, 629-634, 1996). Subsequent studies on muscle restricted insulin receptor knockout mice have shown similar results (Bruning, J.C. *et al., Mol Cell* 2, 559-569, 1998). Interestingly, oxidative soleus muscle of female GLUT4 null mice retained a significant insulin stimulated glucose uptake (Stenbit, A.E. *et al.* , *J Clin Invest* 98, 629-634, 1996). Furthermore, skeletal muscle of GLUT4 null mice maintains normal levels of high energy phosphate pools (Zierath, J.R. *et al., J Biol Chem* 273, 20910-20915, 1998). However, unlike serum glucose level, fed serum free fatty acids and fasted ketone body levels are significantly decreased in GLUT4 null mice (Katz, et al., *Nature* 377, 151-155, 1995). The inventors conducted studies to reveal the mechanism(s) by which skeletal muscle maintains its energetic status and contributes to euglycemia seen in GLUT4 null mice. Several changes in substrate utilization in GLUT4 null mice are seen including activation of a novel insulin responsive and glycemia sensitive glucose transport system accompanied by increased glucose oxidation as well as increased glycolysis at the expense of glycogen synthesis. Altered glucose metabolism in GLUT4 null mice is accompanied by enhanced lipid clearance and increased capacity for oleate oxidation. This increased oxidative capacity is a consequence of mitochondrial hypertrophy and elevated expression of oxidative phosphorylation genes. The overall metabolic profile of GLUT4 null mice resembles

that of an endurance trained athlete. These compensatory metabolic changes prevented the development of diabetes in GLUT4 null mice relevant for designing anti-diabetic strategies.

Clamp studies were performed on conscious 6-9 month old female GLUT4 null and age-/sex-matched control mice. Basal whole body glucose utilization rates were similar between control and GLUT4 null mice, indicating that GLUT4 plays a minimal role in basal glucose disposal (Figure 3A). Hyperinsulinemic clamps were conducted at glucose levels of 100mg/dl and 200mg/dl. Endogenous glucose production was negligible in both control and GLUT4 null mice, indicating normal liver insulin responsiveness. Whole body glucose utilization was decreased 60% in the euglycemic clamp (100 mg/dl), confirming that GLUT4 null mice are severely insulin resistant (Katz, et al., *Nature* 377, 151-155, 1995). Glucose uptake rates into individual tissues were analyzed by the accumulation of phosphorylated $_{14}$C-2-deoxyglucose (2-DOGP) at the end of the clamp. No difference in 2-DOG uptake between GLUT4 null and control mice was seen in kidney, brain, ear, and white adipose tissue (data not shown). Consistent with *in vitro* results reported previously, accumulation of 2-DOGP in GLUT4 null extensor digitorum longus (EDL) was only 15% of control, while soleus muscle was able to maintain 50% of normal insulin stimulated 2-DOG uptake. More interestingly, brown adipose tissue, heart, and diaphragm of GLUT4 null mice retained normal glucose uptake with insulin stimulation (Figure 3B). These results suggest that the novel glucose transport activity originally identified in GLUT4 null soleus is also expressed in these highly oxidative tissues.

Glucose clamp studies performed at 200mg/dl also showed a 50% decrease in whole body glucose utilization rate in GLUT4 null compared to control mice (Figure 3A). However, the increase in glucose utilization rates of GLUT4 null and control mice between the euglycemic and hyperglycemic clamp were very similar (45% of total glucose utilization in control vs. 55% in GLUT4 null) (Figure 3A). Even though EDL continued to be highly insulin resistant (32% of control 2-DOG uptake), brown adipose tissue, heart, diaphragm and soleus of GLUT4 null mice accumulated more

2-DOG than controls (Figure 3B). These results suggest that the novel glucose transport activity in these tissues is enhanced by hyperglycemia.

The fate of glucose entering GLUT4 null tissues through this novel transporter under insulin stimulation and hyperglycemia was compared to that of controls. No insulin stimulated glycogen accumulation was observed in GLUT4 null heart or hindlimb muscle despite insulin stimulated glucose uptake (Figure 3C). However, glycogen levels remained normal in GLUT4 null liver during basal and glucose clamp conditions (Figure 3C). Glucose metabolism was further studied *in vitro* using isolated soleus and EDL muscles. Consistent with the clamp studies, total glucose utilization was 22-26% reduced in GLUT4 null EDL compared to controls under both basal and insulin stimulated conditions (data not shown). Basal glucose metabolism tended to be higher in GLUT4 null soleus than controls (data not shown). A small insulin stimulated increase of total glucose utilization was observed in GLUT4 null soleus muscle as previously reported (Stenbit, A.E. *et al.* , *J Clin Invest* 98, 629-634, 1996). No insulin stimulated increase in glycogen synthesis rate or glycogen content was observed in GLUT4 null muscles (Figure 4A). In control muscles, insulin stimulation caused an increased percentage of glucose partitioning into glycogen synthesis at the cost of glycolysis. Such an increase was not observed in GLUT4 null muscle (Figure 4B). Glucose oxidation rates in isolated GLUT4 null EDL were the same compared to controls. However, glucose oxidation rates were increased 2.3 and 1.6 fold in soleus muscle of GLUT4 null mice compared to controls at basal and insulin stimulated states, respectively. In fact, the percent of glucose oxidized in GLUT4 null soleus and EDL was significantly increased compared to controls (Figure 4B). Glycolysis rates of GLUT4 null EDL were only 20-24% of controls in basal and insulin stimulated states. These results suggest that glucose is preferentially metabolized via glycolysis and oxidation rather than being stored in the form of glycogen in GLUT4 null skeletal muscle.

The effect of GLUT4 ablation on fatty acid metabolism was also studied. In contrast to the decreased insulin stimulated whole body glucose utilization, GLUT4 null mice showed significantly improved fatty acid tolerance by oral olive oil loading

test (Figure 4C). In addition, intramuscular triglyceride content was elevated in GLUT4 nulls compared to controls (EDL: 2.26±0.16 in control vs. 3.09±0.22 in GLUT4 null; soleus: 2.93±0.28 in control vs. 3.70±0.30* in GLUT4 null; n=7 GLUT4 null, n=11 control, *, $p<0.01$, unit: μEq/mg muscle). Fatty acid oxidation was also studied in isolated EDL and soleus muscle of GLUT4 null and control mice in the presence of 5 mM glucose (Figure 4D). A 50% and 30% increased oleate oxidation rates were observed in soleus and EDL muscle of GLUT4 null mice compared to controls (Figure 4D). The increased fatty acid oxidation rates and high triglyceride levels of the GLUT4 null muscle are also characteristics seen in endurance trained athletes (Romijn, J.A. *et al., Am J Physiol* 265, E380-391, 1993; Hurley, B.F. *et al., J Appl Physiol* 60, 562-567, 1986).

To explore the possible molecular mechanism of increased substrate oxidation in GLUT4 null mice, mitochondrial morphology and gene expression of rate limiting enzymes of ATP synthesis were studied. Transmission electron microscopy revealed significant inter-myofibrillar and subsarcolemmelar mitochondrial hypertrophy in both soleus and EDL muscle of GLUT4 null mice (Figure 5A). In contrast to the typical elliptical shape of mitochondria in controls, mitochondria of GLUT4 null muscle appeared to be long and tubular with more densely packed cristae. Mitochondrial hypertrophy was accompanied by 40-80% increased expression of mitochondrial outer membrane proteins in EDL and soleus muscles, respectively (Figure 5B). The expression of representative genes of mitochondrial oxidation such as cytochrome oxidase C and inorganic phosphate carrier was also increased 30% and 50%, respectively (Figure 5C). Such overt mitochondrial hypertrophy as well as increased mitochondrial oxidative gene expression is also observed in skeletal muscle of endurance trained rodents and athletes, suggesting increased substrate oxidative capacity of GLUT4 null mitochondria (Holloszy, J.O. & Booth, *Annu Rev Physiol* 38, 273-291, 1976; Wibom, R. *et al., J Appl Physiol* 73, 2004-2010, 1992; Murakami, T. *et al., Am J Physiol* 267, E388-395, 1994).

White adipose tissue mass and adipocyte size are severely reduced in GLUT4 null mice while fed free fatty acids and fasted ketones levels are significantly

decreased (Katz, et al., *Nature* 377, 151-155, 1995). In view of the diminished triglyceride stores in the GLUT4 null adipose tissue, liver is the most likely organ which can supply the necessary lipid to meet the increased lipid oxidation demands of GLUT4 null muscle. The GLUT4 null liver has unique characteristics that could allow it to supply skeletal muscle with lipids. The GLUT4 null liver has an increased expression of GLUT2 (Katz, et al., *Nature* 377, 151-155, 1995). Such increased expression of GLUT2 protein could result in greater glucose flux into the liver which could be used to synthesize fatty acids. Northern blot analysis revealed increased expression of glucokinase and fatty acid synthase genes in GLUT4 null liver compared to control (Figure 5D). These results suggest that the GLUT4 null liver is capable of taking up large amounts of glucose and subsequently synthesizing triglycerides for utilization by skeletal muscle.

These studies indicate that the GLUT4 null mouse does not develop hyperglycemia because of the unique glucose transport capacity and dramatically altered substrate utilization profile of skeletal muscle. Clamp studies reveal that the GLUT4 null mouse does retain significant insulin stimulated glucose uptake into highly oxidative tissue which is even greater than normal under hyperglycemic conditions implying the activation of a novel low affinity glucose transport activity in GLUT4 null muscle. Insulin stimulated glycogen accumulation was diminished in GLUT4 null muscle, suggesting that GLUT4 is essential for acute glycogen accumulation. In contrast, the novel glucose transport activity identified in this study promotes glycolysis and oxidation rather than glycogen synthesis. Indeed, *in vitro* substrate utilization studies reveal increased oleate and glucose oxidation rates in GLUT4 null muscle when compared to controls. Olive oil loading test further confirmed that GLUT4 null mice have improved fatty acid clearance. GLUT4 null muscle mitochondria hypertrophy and increased gene expression of rate limiting proteins for oxidation provided the cellular machinery for increased substrate oxidation. Despite reduced adipose tissue and adipocyte size in the GLUT4 null mouse (Katz, et al., *Nature* 377, 151-155, 1995), fatty acids could be supplied by increased *de novo* fatty acid synthesis in liver from glucose. In fact, the GLUT4 null

liver exhibits increased expression of GLUT2, glucokinase and fatty acid synthase genes suggesting increased capacity for glucose uptake and subsequent fatty acid synthesis.

Finally, GLUT4 null mice exhibit metabolic similarities to endurance trained athletes (Holloszy, J.O. & Booth, *Annu Rev Physiol* 38, 273-291, 1976; Holloszy, J.O. & Kohrt, W.M., *Annu Rev Nutr* 16, 121-138,1996). They have enlarged hearts, decreased fat depots, increased fatty acid oxidation rates and increased intramuscular triglyceride deposition. The GLUT4 null oxidative muscle is unique in that it maintains both high rates of fatty acid and glucose oxidation. The increased fatty acid oxidation does not compromise glucose oxidation in GLUT4 null mice, a phenomenon commonly observed in diabetes (Randle, P.J., et al., *Lancet* i, 785-789, 1963; Jenkins, et al., *J Clin Invest* 82, 293-299, 1988; Foley, *Diabetes Care* 15, 773-784, 1992). The present study suggests that the positive effect of exercise on improving glycemia in diabetics may, in fact, be due to activation of the novel glucose transport system identified in oxidative tissues of GLUT4 null mice, i.e., GLUTx. GLUTx is a low affinity glucose transporter/sensor expressed in highly oxidative tissues of GLUT4 null mice which may help prevent hyperglycemia characteristics of type II diabetes. These findings suggest that the metabolic programs which allow increased fatty acid oxidation help prevent GLUT4 null mice from developing hyperglycemia. It provides a new target for intervention in treating diabetes.

Discussion

GLUT4 null mice control their glucose within a normal range but they do develop hyperinsulinemia in the fed state (Katz, et al., *Nature* 377: 151-155, 1995). Although their skeletal muscle is insulin resistant, highly oxidative soleus muscle and diaphragm exhibit significant insulin stimulated glucose uptake (Stenbit, et al., *J Clin Invest* 98: 629-634, 1996). The compensatory glucose uptake response is more robust *in vivo* under hyperglycemic clamp conditions. This finding suggests that a glucose sensitive glucose transport system or glucose sensor/receptor-like molecule

is activated in highly oxidative tissues in the absence of GLUT4. This molecule is referred to as GLUTx. Cloning efforts have lead to the isolation of a novel cDNA encoding GLUTx. GLUTx has significant homology to facilitative glucose transporters such as GLUT4 and GLUT1 and has conserved amino acids known to be important in glucose binding. Additionally, GLUTx contains amino acid motifs present only in the glucose sensor/receptors SNF3 and RGT2, and has been detected using in situ hybridization techniques in the cerebellum and hippocampus of GLUT4 null mice, areas corresponding to the "obesity center" of the human brain. These findings suggest that GLUTx functions as a glucose sensor/receptor that assists in maintenance of normal blood glucose and possibly, the control of appetite and regulation of obesity. The functional dominance of GLUT4 may mask the expression/activity of GLUTx under normal conditions, however, GLUTx function may be provoked by certain physiologic or pharmacologic stimuli. As GLUT4 null mice share many features in common with endurance trained athletes (enhanced oxidative capacity, mitochondrial hypertrophy, elevated hexokinase activity, cardiac hypertrophy, reduced serum free fatty acids (fed) and ketones (fasted)), stimuli such as endurance exercise training may activate/upregulate glucose uptake and metabolism via GLUTx. Exercise and reduced glucose uptake through GLUT4 can be viewed as metabolic/nutrient stress signals, thus, GLUTx may signal through the AMP-activated protein kinase (AMPK) cascade. Based upon extensive preliminary results, the inventors hypothesize that TZD treatment stimulates increased muscular glucose uptake and metabolism possibly through GLUTx and/or AMPK. The underlying implication of this hypothesis is that GLUTx activity can be upregulated/activated in humans, not just in GLUT4 null mice. This unique model offers a novel system for studying changes in cellular metabolism and whole body glucose homeostasis which may contribute to the etiology of insulin resistance and type II diabetes. Accordingly, understanding the mechanisms by which GLUT4 null mice avoid hyperglycemia, as well as, alterations in insulin signaling and glucose partitioning in muscle under euglycemic and hyperglycemic conditions, may suggest new therapies for the prevention and treatment of type II diabetes and other

diseases of insulin resistance, as well as the prevention and treatment of obesity and related conditions.

Example 2

In vitro muscle incubations were performed in order to biochemically characterize the transport kinetics of GLUTx and the signal transduction pathways which may regulate it. Using varying concentrations of forskolin, a known activator of adenylate cyclase and competitive inhibitor of glucose transport, 2-deoxyglucose (2-DOG) transport into GLUT4 null soleus muscle was inhibited to a lesser extent than that observed using wild type control soleus muscle. The effect of forskolin on 2-DOG uptake, presumably via GLUTx, was not due to the activation of adenylate cyclase as incubations with bromo-cAMP did not mimic the forskolin results. Furthermore, the effects of forskolin were not attenuated by incubation with dideoxyadenosine, a known antagonist of adenylate cylcase. Combined, these results suggest that forskolin is directly inhibiting GLUTx mediated transport. GLUT4 null muscle was also shown to express a transport system that exhibits increased activity at higher glucose concentrations in *in vivo* studies, suggesting that GLUTx is a low affinity glucose transporter. GLUT4 null soleus was incubated with various concentrations of glucose to confirm this prediction. Indeed, increased 2-DOG transport was measured in GLUT4 null compared to wild type soleus in the presence of 25mM glucose, but not 25mM mannitol. This indicates that GLUTx may exhibit a higher affinity for 2-DOG than for glucose. This effect was shown to be kinetic as 2-DOG transport via GLUTx did not require prior incubation of muscles with 25mM glucose.

Example 3

In vivo studies were performed on mice treated with streptozotocin (STZ) to confirm the *in vitro* characterization of GLUTx. STZ is a toxin that destroys insulin secreting beta cells resulting in insulinopenia and diabetic hyperglycemia. GLUT4 null and wild type mice were equally sensitive to STZ-treatment (glycemia 400-500 mg/dl or 22-25 mM), however, GLUT 4 null mice were more insulin resistant as

shown by insulin tolerance tests. Surprisingly, 2-DOG uptake into soleus muscle in the presence of 25 mM glucose was severely blunted under basal and insulin stimulated conditions in GLUT 4 null compared to wild type. This result suggests that GLU4 null mice, which express the low affinity transporter GLUTx, may be more sensitive to glucose toxicity noted in diabetic hyperglycemia and/or that GLUTx may transport STZ, thereby preferentially damaging GLUT4 null cells. STZ is known to be transported into cells by GLUT2, another low affinity glucose transporter, therefore it is likely that GLUTx also mediates transport of this toxin. In order to further characterize this newly identified transport activity, muscles were incubated with inhibitors (wortmannin, vanadate or okadaic acid) that alter glucose transport by affecting different levels of the insulin signaling pathway. Preincubation of muscle with these compounds did not alter 2-DOG transport in GLUT4 null mice. Immunoblot analysis revealed that total and phosphorylated Akt/PKB levels were significantly elevated in GLUT4 null muscle under basal and insulin stimulate conditions *in vivo*. Taken together, these results implicate the involvement of Akt/PKB levels, independent of PI3 kinase, in the activation of the novel, glycemia sensitive transporter (GLUTx) and the compensatory mechanisms that prevent the development of diabetes in GLUT4 null mice.

Example 4

2-N-4-1-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-bis(d-mannose-4-yloxy)-2-propylamine (ATB-BMPA) exofacial photolabel was used to determine if increased cell surface GLUT1 content accounts for insulin stimulated glucose uptake in soleus muscle from female GLUT4 null mice. Insulin increased the rate of 2-DOG uptake 2.8 fold in wild type soleus muscle, and 2.1 fold in GLUT4 null soleus muscle. Cytochalasin B, an inhibitor of GLUT1 and GLUT4 mediated glucose transport, inhibited 2-DOG uptake in the insulin-stimulated state by more than 95% in wild type and GLUT4 null soleus muscle. Addition of 35mM fructose to the incubation media was without effect on 3-O-methylglucose transport activity in wild type and GLUT4 null soleus muscle under insulin stimulated conditions. In contrast,

incubation of soleus muscles in the presence of 35 mM glucose inhibited 3-O-methylglucose transport in the insulin stimulated state (20nM) by 65% in wild type, and 100% in GLUT4 null mice. In wild type soleus muscle, cell surface GLUT4 content was increased by 2.8 fold under insulin stimulated conditions, and this increase corresponded with the increase in 2-DOG uptake. No detectable cell surface GLUT4 was observed in soleus muscle from female GLUT4 null mice under either basal or insulin stimulated conditions. Under basal conditions, cell surface GLUT1 content was equally abundant in wild type and GLUT4 null mice, with no further increase noted in either genotype with insulin exposure. Accordingly, insulin stimulated glucose activity in female GLUT-4 null soleus muscle is mediated by a facilitative transport process that is glucose and cytochalasin B inhibitable. Furthermore, increased cell surface GLUT1 content does not appear to compensate for the lack of GLUT4 in soleus muscle from female GLUT4 null mice.

Example 5

To determine the effect of GLUT4 ablation on post-exercise glucose transport and glycogen synthesis, GLUT4 null and wild type mice were studied immediately after a 3 hour swim bout, or after carbohydrate re-feeding. In fasted or fed wild type mice, insulin and swimming independently increased 2-DOG uptake 2-fold ($p<0.05$) in extensor digitorum longus (EDL) muscle. In contrast, insulin did not increase glucose transport in EDL muscle from either fasted or fed GLUT4 null mice. Acute swimming led to a 2-fold increase ($p<0.05$) in glucose transport in EDL muscle from fed GLUT4 null mice, with no effect noted in muscle from fasted GLUT4 null mice. Electrical stimulation of isolated EDL muscle from fed GLUT4 null mice led to a 1.6-fold increase in glucose transport activity ($p<0.01$). However, the magnitude of the contraction-induced increase in glucose transport was 4-fold greater in wild type muscle ($p<0.05$). The exercise-associated glucose transport activity in GLUT4 null EDL muscle was not accompanied by increased cell surface GLUT1 content. Glycogen content in gastrocnemius muscle was similar between fed wild type and GLUT4 null EDL. Swimming reduced muscle glycogen content by around 50%

($p<0.05$) in both wild type and GLUT4 null mice. After 5 hour carbohydrate re-feeding muscle glycogen content was fully restored in wild type mice ($p<0.05$), whereas no significant gain was noted in GLUT4 null mice. After 24 hour carbohydrate re-feeding, muscle glycogen levels in GLUT4 null mice were restored to pre-exercise fed levels. Glycogen synthase protein expression and fractional activity were similar between wild type and GLUT4 null mice. Regardless of genotype, liver glycogen levels were restored to non-exercised levels after 5 hr carbohydrate re-feeding. It was concluded that GLUT4 is not essential for glycogen repletion, since muscle glycogen levels in previously exercised GLUT4 null mice were completely restored after 24 hr carbohydrate re-feeding. Further, evidence is provided that an exercise induced increase in glucose transport activity may be mediated by GLUT4 and GLUT1 independent mechanisms, which may comprise GLUTx activity.

Example 6

The inventors hypothesized the existence of a membrane protein structurally related to glucose transporters which may transport glucose in the absence of GLUT4 in null mice, i.e. GLUTx. This membrane protein could be similar to SNF3 or RGT2 of *Saccharomyces* which are similar in structure to glucose transporters and can transport glucose but not in sufficient quantities to sustain cell growth and division (Ozcan, et al., *Proc Natl Acad Sci U S A* 93: 12428-32, 1996; Ozcan, et al., *Embo J* 17: 2566-73, 1998). Their main function is to transmit a signal of low glucose (SNF3) or high glucose (RGT2) so that appropriate glucose transporters can be expressed (Ozcan, et al., *Proc Natl Acad Sci U S A* 93: 12428-32, 1996; Ozcan, et al., *Embo J* 17: 2566-73, 1998). The inventors generated a series of degenerate oligonucleotide primers corresponding to conserved areas of known glucose transporters that encompassed sequences known to be important for glucose transport (Hellwig, et al., *Biochim Biophys Acta* 1111: 178-84, 1992; Joost, et al., *Exp Clin Endocrinol* 102: 434-8, 1994; Olson, A.L. and Pessin, J.E., *Annu Rev Nutr* 16: 235-56, 1996). RT-PCR analysis was performed using GLUT4 null muscle RNA with different combinations of 5' and 3' primers. From this analysis, the inventors

identified a novel glucose transporter-like sequence, GLUTx. Partial sequence of GLUTx corresponding to the area between the 10th transmembrane domain and the carboxy-terminus of known mammalian GLUTs is shown in Figure 6. This area includes tryptophans 387 and 412 which are known to be important in glucose sensing and transport (Schurmann, et al., *Biochem J* 290: 497-501, 1993) and a highly conserved area in all transporters just outside the 12th transmembrane domain (PETxG). Approximately 45% sequence similarity is noted between GLUT4 and GLUTx in the region shown in Figure 6. GLUTx also contains a stretch of amino acid residues (CLFIA) homologous to RGT2 and SNF3 and not found in any other glucose transporter in the mouse (Fig. 7). Approximately 40% sequence similarity is noted between GLUTx and RGT2/SNF3 in the region shown in Figure 7. Preliminary northern blot analysis identified an approximately 2.4 kb major mRNA (and 5.0 kb and 1.7 kb minor mRNAs) in tissues such as skeletal muscle, diaphragm, heart, fat, spleen, brain, and liver which is of low abundance, and in testis which is of significant abundance (Figure 8). From this preliminary analysis, GLUTx appears to be upregulated in soleus and un-regulated in WAT from GLUT4 null mice (-) compared to controls (+). These results are in strong agreement with the observed insulin stimulated increase in glucose uptake in GLUT4 null soleus and lack of a compensatory glucose uptake response in WAT.

Rat EST H34451 has a significant similarity to RGT2 and glucose transporters, including a stretch of amino acids, CFLIA, contained in the 11th membrane spanning domain. This sequence is found only in RGT2 and SNF3 and not in the yeast glucose transporters. The sequence H34451 was used to make a probe for hybridizing to a Northern of control and null tissues, including heart, diaphragm, testis and fat. RT-PCR of GLUT4 null and normal tissues using oligos specific to the H34451 sequence resulted in a band with a sequence almost identical to the rat sequence. Further searching of the database revealed several sequences with similarities to glucose transporters. Sequencing the full length of one of the human ESTs, R11726, showed that it too contained the unique sequence of CFLIA and contained significant similarity to H34451. Human EST H09414 was found in a subsequent search to

overlap R11726 at the 5' end. This 5' end was extended further when EST AA293722 was revealed to overlap H09414 at the 5'end. These sequences have subsequently been grouped with R11726 into a human unigene that has similarity GLUT3. Using the methods identified above, the inventors obtained the partial nucleotide sequences (together with deduced amino acid sequences) encoding for human, rat and mouse GLUTx. The partial human nucleotide sequence for human GLUTx is shown in Figure 10, with the corresponding amino acid sequence of Figure 9. Partial rat nucleotide sequence encoding for rat GLUTx is shown in Figure 12, with the corresponding amino acid translation as shown in Figure 11. The partial nucleotide and corresponding amino acid sequence of mouse GLUTx is shown as Figures 14 and 13, respectively.

Example 7

GLUT4 null skeletal muscle can utilize glucose in the absence of GLUT4. It is likely that intracellular alterations of glucose utilization may occur to compensate for the decrease in glucose transport as a result of GLUT4 ablation. To test this hypothesis, hexokinase (HK) activity was measured in hindlimb muscle spectrophotometrically as previously described (Tsao, et al., *J Biol Chem* 271: 14959-14963, 1996) by coupling the HK reaction to the glucose-6-phosphate dehydrogenase reaction. HKII activity was separated from HKI activity by their different stabilities at 45°C. HKII activity from GLUT4 null muscle was increased 52% (mU). No difference in HKI activity was observed. As HKII is the major HK isoform in skeletal muscle, total HK activity in GLUT4 null muscle was increased by 52% vs. controls. It was shown previously that HKII expression is positively regulated by insulin (Postic, et al., *Diabetes* 42: 922-929, 1993). Furthermore, HK activity increases with endurance exercise training muscle (Nakatani, et al., *J Appl Physiol* 82: 711-5, 1997).

To address the concern that 2-DOG transport activity seen in GLUT4-null soleus was an artifact due to increased HK II activity, transport studies were conducted using the non-phosphorylated glucose analogue 3-O-methylglucose (3-

MG). Results are shown in Fig. 15. GLUT4-null soleus exhibited 3-MG uptake rates that were insulin-sensitive and significant in magnitude. These results obtained using 3-MG were similar to those obtained using 2-DOG. As 3-MG is not phosphorylated following transport, the significant and insulin-sensitive glucose uptake observed in GLUT4-null soleus cannot be attributed to increased HK II expression. In addition, as shown in Figs. 16-18, isolated GLUT4 null soleus exhibited considerable total glucose utilization (glycogen synthesis and glycolysis combined). Furthermore, it has been shown previously in transgenic mice studies that a 2- to 4-fold increase in skeletal muscle HK II expression is required to achieve even a slight increase in 2-DOG uptake (Chang, et al., *J Biol Chem* 271: 14834-14839, 1996). As a result, it is not likely that the comparatively smaller increase in HK II activity (noted above) in GLUT4 null muscle can account for the significant 2-DOG uptake observed in soleus but not EDL. It has also been previously demonstrated that 2-DOG uptake in rat soleus remains linear for 60 min at a 2-DOG concentration of 8 mM (Hansen, et al., *J Appl Physiol* 76: 979-985, 1994). This indicates that glucose phosphorylation is not rate-limiting under those conditions. The concentration of 2-DOG in our studies was 0.1 mM, far below that in the previous study. It is unlikely that glucose phosphorylation is rate-limiting in our experimental conditions.

Example 8

To assess the consequences of GLUT4 ablation on intracellular glucose metabolism, soleus and EDL muscles from control and GLUT4 null mice were incubated in media containing 5 mM glucose in the presence and absence of insulin. Both U-[^{14}C]- and 5-[^{3}H]-glucose tracers were included in the media as previously described (Ashcroft, et al., *Biochem J* 126: 525-32, 1972; Le Marchand-Brustel, et al., *Am J Physiol* 234: E348-E358, 1978; Cuendet, et al., *J Clin Invest* 58: 1078-1088, 1976; Heydrick, et al., *Am J Physiol* 268: E604-12, 1995). Results of these studies are presented in Figs. 16-18. In accordance with decreased glucose uptake rates, null EDL muscles exhibited decreased glycolysis rates (Fig. 16). Insulin stimulated glycogen synthesis rates in null EDL were reduced to the same extent as glycolysis

(Fig. 17). However, glycogen content in null EDL under insulin action was decreased only 26%. Interestingly, while basal glycogen synthesis rates in null EDL were decreased 40%, basal glycogen content was normal. Surprisingly, glucose oxidation rates in null and control EDL were similar under basal and insulin stimulation. In contrast with EDL, null soleus exhibited normal glycolysis rates. Basal glycogen synthesis rates in null soleus were also normal. Consistent with glucose uptake, insulin stimulated glycogen synthesis rates were decreased by 40% in null soleus. However, null soleus contained normal amounts of glycogen under basal and insulin stimulation. In addition, null soleus exhibited a 40% increase in glucose oxidation rates under both basal and insulin stimulated conditions (Fig. 18). Thus, even though insulin stimulated glucose uptake was decreased, compensatory adaptations have taken place in null soleus to normalize glycolysis. It is likely that GLUTx expression in null soleus muscle contributes to the compensatory responses noted above. Further evidence of compensatory adaptations includes increases in basal and insulin stimulated oxidation rates. As free fatty acid oxidation rates were also increased, it is likely that the overall oxidative capacity was increased in null soleus and EDL. Similar compensatory adaptations have been observed in insulin resistant animals treated with TZD (Oakes, et al., *Diabetes* 43: 1203-10, 1994; Okuno, et al., *Metabolism* 46: 716-21, 1997). TZDs can normalize previously impaired glucose utilization by preferential enhancement of glycolysis and glucose oxidation, in addition to restoring glucose uptake (Oakes, et al., *Diabetes* 43: 1203-10, 1994; Okuno, et al., *Metabolism* 46: 716-21, 1997).

Several observations lend further support to the concept that GLUT4 null muscle activates a metabolic/genetic program similar to endurance trained muscles. First, expression of cytochrome oxidase (COX IV) and inorganic phosphate carrier (PiC) genes is increased in GLUT4 null muscle. Second, measurement of uncoupling protein gene expression has revealed a 45% decrease in UCP3 mRNA and no change in UCP2 mRNA. Lastly, transmission electron microscopy has revealed extensive mitochondrial hypertrophy in GLUT4 null muscle. Taken together these data suggest that there is reduced leaking of protons from GLUT4 null mitochondria and

increased coupling of the electron transport chain and oxidative phosphorylation in order to maintain high energy phosphate stores. Muscular mitochondrial hypertrophy is noted with endurance training (Holloszy, J.O. and Booth, F.W., *Annu Rev Physiol* 38: 273-291, 1976). Interestingly, mitochondrial hypertrophy is also noted in yeast which have been starved for glucose (Lin, et al., *Arch Biochem Biophys* 160: 458-464, 1974; Mian, et al., *J Bacteriol* 115: 876-881, 1973). The commonality of metabolic stress is noted with endurance training and glucose starvation, therefore, GLUT4 null muscle may be in a similar 'stressed' state which activates or enhances GLUTx expression. All of the above lend further support to the contention that GLUT4 null mice have made metabolic/genetic adaptations similar to those achieved by endurance trained athletes.

The inventors have hypothesized that similar metabolic/genetic alterations are induced by TDZ treatment. Indeed GLUT4 null muscle appears to have activated PPARg as evidenced by ectopic/unpredicted expression of an aP2-GLUT4 transgene in muscle and fat. As the adipocyte specific aP2 gene is activated by the transcription factor PPARg, expression of the adipose-restricted aP2-GLUT4 transgene (provided by Dr. B.B. Kahn; (Shepherd, et al., *J Biol Chem* 268: 22243-22246, 1993) in muscle of GLUT4 null mice was unexpected. Additionally, PPARg is upregulated in adipose tissue of GLUT4 null mice which is further suggestive that GLUT4 null mice have activated PPARg by endogenous mechanisms.

Example 9

Preliminary in situ hybridization studies indicate that GLUTx is expressed in the cerebellum and hippocampus of GLUT4 null mice, the same areas where GLUT 4 is expressed in wild type mice. These studies suggest GLUTx may function as a glucose sensor or receptor in the "obesity center" of the brain, and may therefore provide an attractive target for the study and treatment of obesity.

Example 10

A polyclonal antibody was generated to the last 11 amino acids of the

carboxy-terminus of the GLUTx protein. These amino acids are LEQITAHFEGR. The antibody was used in Western blot analysis of different tissues from GLUT4 null and wild type mice and of mammary tumors induced by the mouse mammary tumor virus. A specific immunoreactive protein was found to be about 32.6 kD in testis, heart, fat, liver, diaphragm, and soleus muscle in both GLUT4 null and wild type mice. Further analysis revealed that GLUTx protein appears to be more abundant in GLUT4 null liver and testis than in the same wild type tissues. In contrast, the GLUT4 null fat seems to express less GLUTx protein than wild type fat. The Western blot analysis of the mouse mammary tumor showed an approximately 32.6 kD protein while normal mouse mammary tissue did not appear to have a band in this area.

Northern blot analysis using a probe to mouse GLUTx shows a band at about 2.4kb. As noted above, the GLUTx mRNA expression varies among tissues of the GLUT4 null and wild type mice and also between GLUT4 null and wild type tissue. In collaboration with Dr. Sylvie Haugel-de Mouzon (INSERM, Paris), a GLUTx probe was used on Northern blots of tissues from normal and streptozoticin diabetic rats and tissue from rats subjected to hyperglycemic clamps to determine the expression of GLUTx mRNA in various metabolic milieu. The mRNA expression of GLUTx was highest in the testis of normal and streptozotocin diabetic rats with brain and placenta expression about one half as much. The liver expresses about one-tenth the amount seen in the testis in the normal rat. GLUTx mRNA expression in testis and brain was the same in normal as in streptozotocin diabetic and hyperglycemic clamp rats, however the livers and placentas of the diabetic and hyperglycemic clamp rats showed a 2 to 3 fold upregulation of GLUTx mRNA expression when compared to normal rats. Streptozotocin is a pancreatic beta cell toxin that induces hyperglycemia and insulinopenia. Since GLUTx mRNA was increased similarly in the streptozotocin treated rats and the rats clamped under hyperglycemic conditions, GLUTx mRNA appears to be regulated by high glucose (and not high insulin). Results of this series of experiments confirm our hyperglycemic clamp results which suggested that GLUTx is glycemia sensitive.

A hydropathy plot was performed on the primary sequence of GLUTx and 12 transmembrane domains identified. All known facilitated GLUTs have 12 transmembrane domains. From this, it can be predicted that the amino terminus of GLUTx may be only approximately 20 amino acids long. In addition to 5' RACE cloning, a mouse BAC clone was isolated that contains the GLUTx genomic sequences. Restriction fragments have been subcloned from the GLUTx BAC and will be sequenced to elucidate the 5' most amino terminal sequences of the GLUTx protein.

All publications mentioned herein above are hereby incorporated by reference in their entirety. While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of the disclosure that various changes in form and detail can be made without departing from the true scope of the invention in the appended claims.

What is Claimed is:

1. A purified and isolated nucleic acid encoding GLUTx.

2. The nucleic acid of Claim 1 that is derived from a human, a mouse or a rat.

3. The nucleic acid of Claim 2 that is derived from a human.

4. The nucleic acid of Claim 2 that is derived from mouse.

5. The nucleic acid of Claim 2 that is derived from a rat.

6. The nucleic acid of Claim 3 which encodes the amino acid sequence for GLUTx shown in Figure 10.

7. The nucleic acid of Claim 6, having the nucleotide sequence for GLUTx as shown in Figure 9.

8. The nucleic acid of Claim 4 which encodes the amino acid sequence for GLUTx shown in Figure 12.

9. The nucleic acid of Claim 8, having the nucleotide sequence for GLUTx as shown in Figure 11.

10. The nucleic acid of Claim 5 which encodes the amino acid sequence for GLUTx shown in Figure 14.

11. The nucleic acid of Claim 10, having the nucleotide sequence for GLUTx as shown in Figure 13.

12. A vector comprising a nucleic acid encoding GLUTx.

13. A host cell transformed by the vector of Claim 12.

14. A method for producing recombinant GLUTx comprising growing a host cell transformed with the vector of Claim 12 and isolating the recombinant GLUTx from said culture.

15. The method of Claim 14, wherein said host cell is a prokaryotic cell.

16. The method of Claim 14, wherein said host cell is a eukaryotic cell.

17. A purified GLUTx protein or an analogue thereof.

18. The purified GLUTx protein of Claim 17 which is recombinantly produced.

19. A nucleic acid probe which hybridizes to nucleic acid encoding GLUTx.

20. The nucleic acid of Claim 1 having one or more mutations.

21. The nucleic acid of Claim 20, wherein the mutations are selected from the group consisting of a point, insertion, rearrangement or deletion mutation.

22. An agent that binds to the protein of Claim 17.

23. The agent of Claim 22, which is an antibody, a peptide, a protein, a nucleic acid, a drug, or antisense nucleic acid.

24. The agent of Claim 23 which is an agonist of GLUTx.

25. An isolated nucleic acid comprising a nucleotide sequence which is at least 80% homologous with the nucleic acid sequence of Claim 1.

26. An isolated nucleic acid comprising a nucleotide sequence which is at least 85% homologous with the nucleic acid sequence of Claim 1.

27. An isolated nucleic acid comprising a nucleotide sequence which is at least 90% homologous with the nucleic acid sequence of Claim 1.

28. An isolated nucleic acid comprising a nucleotide sequence which is at least 95% homologous with the nucleic acid sequence of Claim 1.

29. An isolated nucleic acid comprising a nucleotide sequence which is at least 98% homologous with the nucleic acid sequence of Claim 1.

30. A non-human, transgenic animal model comprising a nucleic acid encoding GLUTx incorporated into some of the somatic cells of said animal.

31. The animal model of Claim 30, wherein said nucleic acid encodes a functional GLUTx protein.

32. The animal model of Claim 31, wherein said nucleic acid has one or more mutations.

33. An agent that binds to the nucleic acid of Claim 1.

34. An agent that enhances the expression of the nucleic acid of Claim 1.

35. The agent of Claim 34, which is a transcription factor, an activator, or a repressor.

36. A method for screening for an agent that binds to the nucleic acid of Claim 1 comprising contacting the nucleic acid with an agent of interest and assessing the ability of the agent to bind to the nucleic acid.

37. A method for screening for an agent that enhances the expression of the nucleic acid of Claim 1 comprising contacting a cell transformed with a vector comprising the nucleic acid, and assessing the effect of the agent on expression of the nucleic acid.

38. A method for screening for an agent that binds to the protein of Claim 17 comprising contacting the protein with an agent of interest and assessing the ability of the agent to bind to the protein.

39. A method of treating type-II diabetes comprising administering to a subject a nucleic acid molecule comprising a nucleotide sequence encoding the amino acid sequence of Figure 10, such that said nucleic acid sequence is expressed in target cells of the patient thereby alleviating the type-II diabetes.

40. The method of Claim 39, wherein the nucleic acid is transfected into target cells by infection with a replication defective virus or by transfection with a liposome comprising said nucleic acid.

41. The method of Claim 40, wherein the target cells are skeletal muscle cells.

42. The method of Claim 41, wherein the nucleic acid molecule comprises the nucleotide sequence of Figure 9.

43. A method of treating type-II diabetes in a subject comprising administering to a subject the agent of Claim 24 or 34 with a physiologically acceptable carrier in an amount effective to treat type-II diabetes in the subject.

NOVEL GLUCOSE TRANSPORTER/SENSOR PROTEIN AND USES THEREOF

Abstract of the Disclosure

The present invention provides a purified and isolated nucleic acid encoding a GLUTx glucose transporter/receptor/sensor protein. The present invention also provides a vector comprising nucleic acid encoding GLUTx, a host cell transformed with the vector, and a method for producing recombinant GLUTx protein. In addition, the present invention also provides a purified GLUTx protein. Also provided by the present invention are nucleic acid probes and mixtures thereof specific for GLUTx nucleic acid and antibodies immunoreactive with GLUTx. The present invention also provides a methods for screening for agents which bind to the GLUTx protein and the nucleic acid encoding the GLUTx. Also provided is a method of promoting euglycemic homeostasis in a subject comprising introducing nucleic acid encoding GLUTx to the subject so that GLUTx is expressed in an amount effective to promote euglycemic homeostasis in a subject. Finally, the present invention provides a non-human, transgenic model for GLUTx expression.

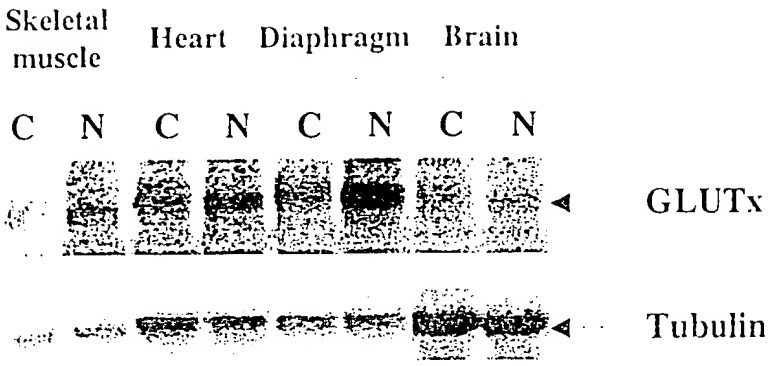
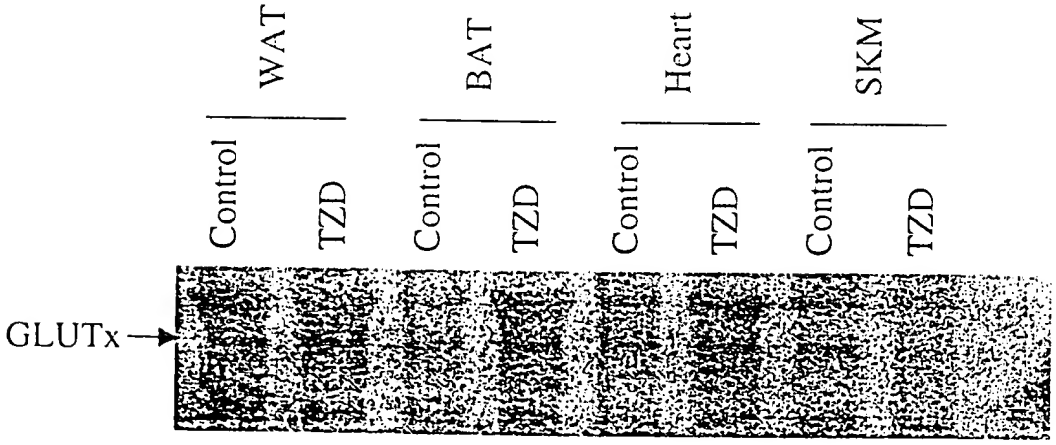

Skeletal muscle
Heart
Diaphragm
Brain
C N C N C N C N
GLUTx
Tubulin

FIG. 1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BAT
Heart
SKM
Control
TZD
Control
TZD
Control
TZD
Control
TZD
GLUTx

FIG. 2

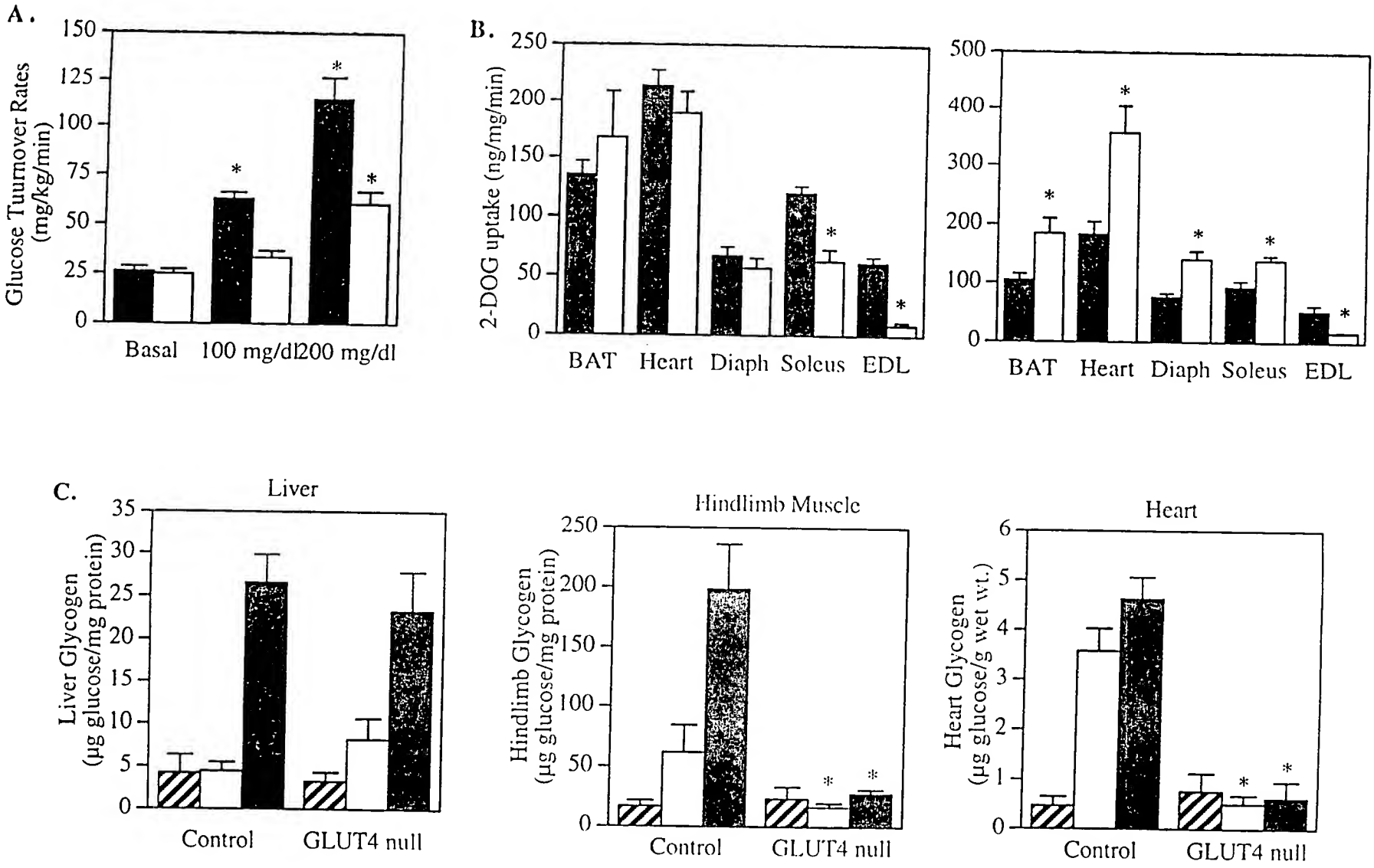
A.
Glucose Tuurnover Rates (mg/kg/min)
150
125
100
75
50
25
0
Basal
100 mg/dl
200 mg/dl
B.
2-DOG uptake (ng/mg/min)
250
200
150
100
50
0
BAT
Heart
Diaph
Soleus
EDL
500
400
300
200
100
0
BAT
Heart
Diaph
Soleus
EDL
C.
Liver
Liver Glycogen (µg glucose/mg protein)
35
30
25
20
15
10
5
0
Control
GLUT4 null
Hindlimb Muscle
Hindlimb Glycogen (µg glucose/mg protein)
250
200
150
100
50
0
Control
GLUT4 null
Heart
Heart Glycogen (µg glucose/g wet wt.)
6
5
4
3
2
1
0
Control
GLUT4 null

FIG. 3

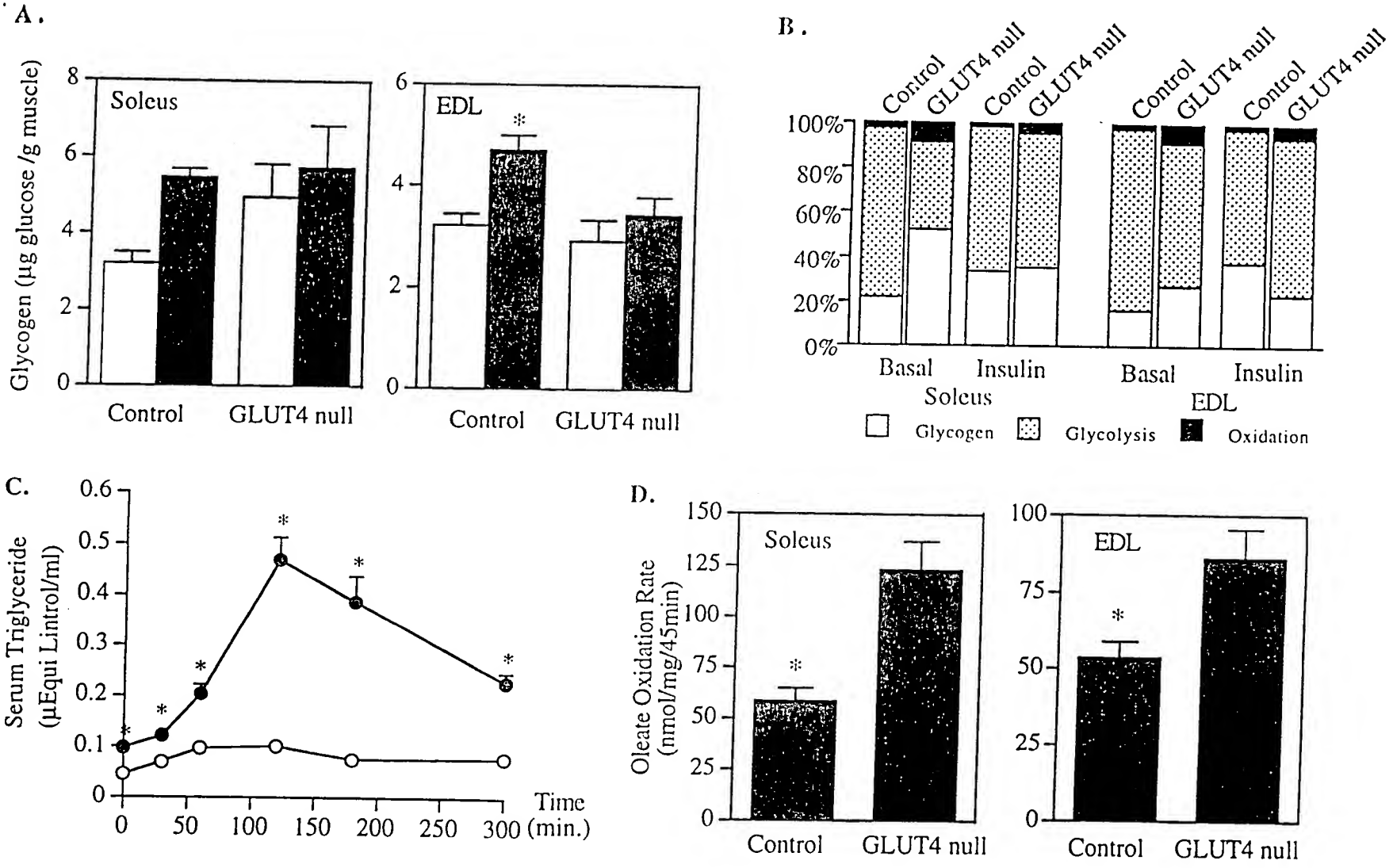
A.
Glycogen (μg glucose /g muscle)
Soleus
EDL
Control
GLUT4 null
B.
100%
80%
60%
40%
20%
0%
Basal
Insulin
Soleus
EDL
Glycogen
Glycolysis
Oxidation
C.
Serum Triglyceride (μEqui Lintrol/ml)
Time (min.)
D.
Oleate Oxidation Rate (nmol/mg/45min)

FIG. 4

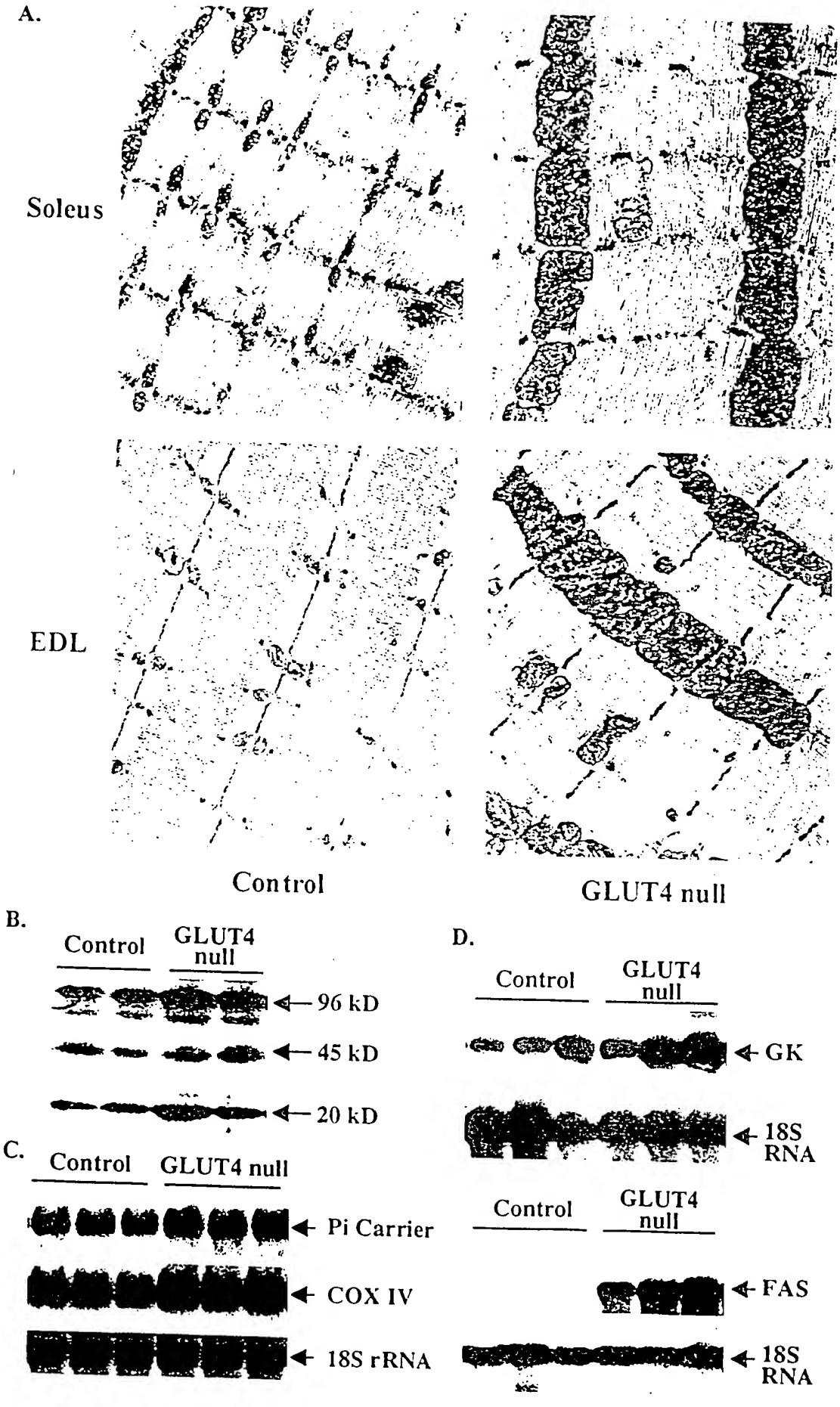
A.
Soleus
EDL
Control
GLUT4 null
B.
Control
GLUT4 null
96 kD
45 kD
20 kD
C.
Control
GLUT4 null
Pi Carrier
COX IV
18S rRNA
D.
Control
GLUT4 null
GK
18S RNA
Control
GLUT4 null
FAS
18S RNA

FIG. 5

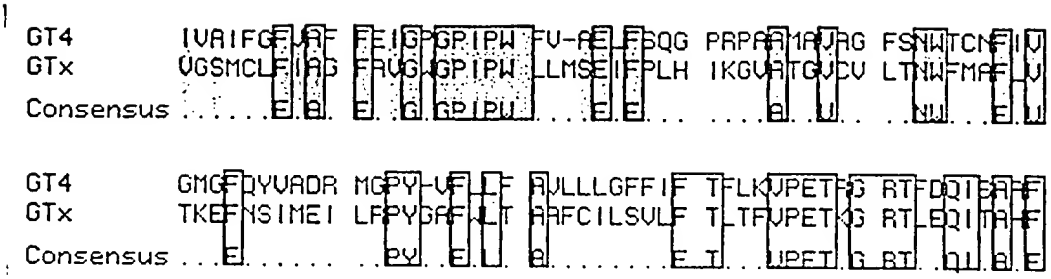
GT4
GTx
Consensus
GT4
GTx
Consensus

FIG. 6

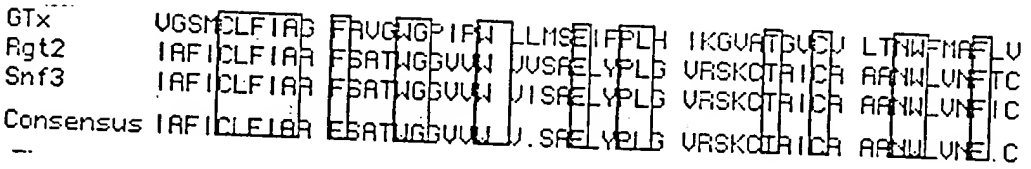
GTx
Rgt2
Snf3
Consensus

FIG. 7

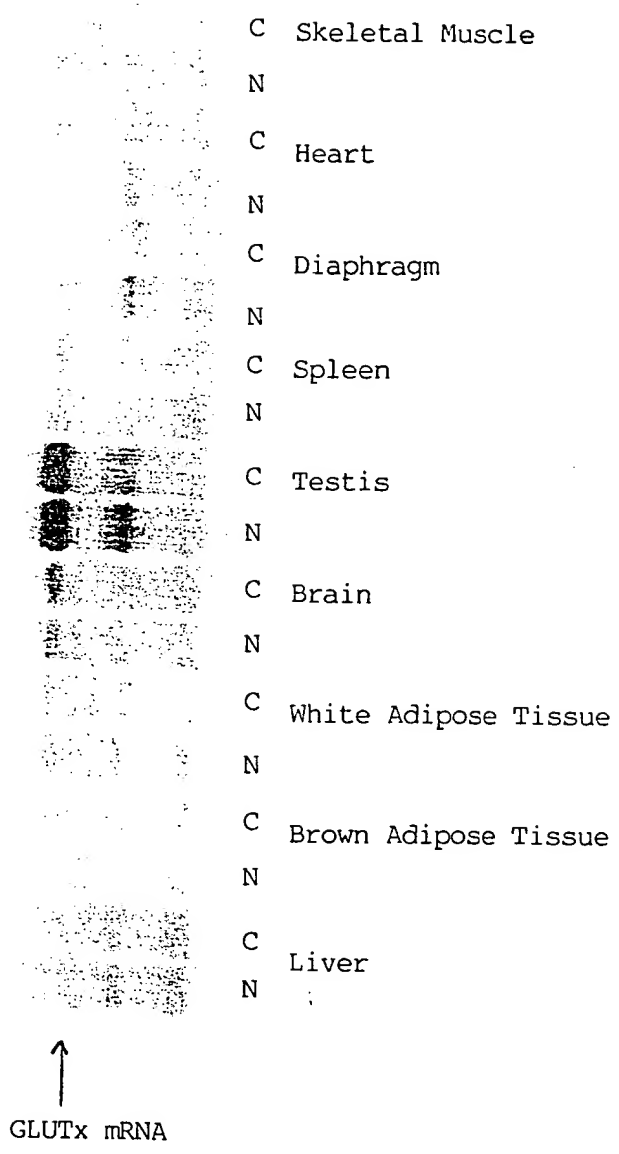
C Skeletal Muscle
N
C Heart
N
C Diaphragm
N
C Spleen
N
C Testis
N
C Brain
N
C White Adipose Tissue
N
C Brown Adipose Tissue
N
C Liver
N
GLUTx mRNA

FIGURE 8

HUMAN nucleic acid sequence

AACTTGCGGCCGCCGCGTCTTCCTCGCCGCCTTCGCCGCTGCCCTG
GGCCCACTCAGCTTCGGCTTCGCGCTCGGCTACAGCTCCCCGGCCA
TCCCTAGCCTGCAGCGCGCCGCGCCCCCGGCCCCGCGCCTGGACG
ACGCCGCCGCCTCCTGGTTCGGGGCTGTCGTGACCCTGGGTGCCG
CGGCGGGGGGAGTGCTGGGCGGCTGGCTGGTGGACCGCGCCGGGC
GCAAGCTGAGCCTCTTGCTGTGCTCCGTGCCCTTCGTGGCCGGCTT
TGCCGTCATCACCGCGGCCCAGGACGTGTGGATGCTGCTGGGGGG
CCGCCTCCTCACCGGCCTGGCCTGCGGTGTTGCCTCCCTAGTGGCC
CCGGTCTACATCTCCGAAATCGCCTACCCAGCAGTCCGGGGGTTGC
TCGGCTCCTGTGTGCAGCTAATGGTCGTCGTCGGCATCCTCCTGGC
CTACCTGGCAGGCTGGGTGCTGGAGTGGCGCTGGCTGGCTGTGCT
GGGCTGCGTGCCCCCCTCCCTCATGCTGCTTCTCATGTGCTTCATG
CCCGAGACCCCGCGCTTCCTGCTGACTCAGCACAGGCGCCAGGAG
GCCATCGCCCTGCGGTTCCTGTGGGGCTCCGAGCAGGGCTGGGAA
GACCCCCCCATCGGGGCTGAGCAGAGCTTTCACCTGGCCCTGCTGC
GGCAGCCCGGCATCTACAAGCCCTTCATCATCGGTGTCTCCCTGAT
GGCCTTCCAGCAGCTGTCGGGGGTCAACGCCGTCATGTTCTATGCA
GAGACCATCTTTGAAGAGGCCAAGTTCAAGGACAGCAGCCTGGCC
TCGGTCGTCGTGGGTGTCATCCAGGTGCTGTTCACAGCTGTGGCG
GCTCTCATCATGGACAGAGCAGGGCGGAGGCTGCTCCTGGTCTTG
TCAGGTGTGGTCATGGTGTTCAGCACGAGTGCCTTCGGCGCCTACT
TCAAGCTGACCCAGGGTGGCCCTGGCAACTCCTCGCACGTGGCCAT
CTCGGCGCCTGTCTCTGCACAGCCTGTTGATGCCAGCGTGGGGCT
GGCCTGGCTGGCCGTGGGCAGCATGTGCCTCTTCATCGCCGGCTT
TGCGGTGGGCTGGGGGCCCATCCCCTGGCTCCTCATGTCAGAGAT
CTTCCCTCTGCATGTCAAGGGCGTGGCGACAGGCATCTGCGTCCTC
ACCAACTGGCTCATGGCCTTTCTCGTGACCAAGGAGTTCAGCAGCC

FIG. 9

TCATGGAGGTCCTCAGGCCCTATGGAGCCTTCTGGCTTGCCTCCGC
TTTCTGCATCTTCAGTGTCCTTTTCACTTTGTTCTGTGTCCCTGAA
ACTAAAGGAAAGACTCTGGAACAAATCACAGCCCATTTTGAGGGGC
GATGACAGCCACTCACTAGGGGATGGAGCAAGCCTGTGACTCCAA
GCTGGGCCCAAGCCCAGAGCCCCTGCCTGCCCCAGGGGAGCCAGA
ATCCAGCCCCTTGGAGCCTTGGTCTGCAGGGTCCCTCCTTCCTGTC
ATGCTCCCTCCAGCCCATGACCCGGGGCTAGGAGGCTCACTGCCTC
CTGTTCCAGCTCCTGCTGCTGCTCTGAGGACTCAGGAACACCTTCG
AGCTTTGCAGACCTGCGGTCAGCCCTCCATGCGCAAGACTAAAGCA
GCGGAAGAGGAGGTGGGCCTCTAGGATCTTTGTCTTCTGGCTGGA
GGTGCTTTTGNAGGTTGGGTGCTGGGCATTCGGTCGCTCCTCTCAC
GCGGCTGCCTTATCGGGAAGGAAATTTGTTTGCCAAATAAAGACGT
GACACAGAAAATCAAAAAAAAAAAAAAAAAAATTCC

FIG. 9 cont.

HUMAN amino acid sequence

RRVFLAAFAAALGPLSFGFALGYSSPAIPSLQRAAPPAPRLDDAAASW
FGAVVTLGAAAGGVLGGWLVDRAGRKLSLLLCSVPFVAGFAVITAAQ
DVWMLLGGRLLTGLACGVASLVAPVYISEIAYPAVRGLLGSCVQLMV
VVGILLAYLAGWVLEWRWLAVLGCVPPSLMLLLMCFMPETPRFLLTQ
HRRQEAIALRFLWGSEQGWEDPPIGAEQSFHLALLRQPGIYKPFIIGV
SLMAFQQLSGVNAVMFYAETIFEEAKFKDSSLASVVVGVIQVLFTAVA
ALIMDRAGRRLLLVLSGVVMVFSTSAFGAYFKLTQGGPGNSSHVAIS
APVSAQPVDASVGLAWLAVGSMCLFIAGFAVGWGPIPWLLMSEIFPL
HVKGVATGICVLTNWLMAFLVTKEFSSLMEVLRPYGAFWLASAFCIF
SVLFTLFCVPETKGKTLEQITAHFEGR*QPLTRGWSKPVTPSWAQAQ
SPCLPQGSQNPAPWSLGLQGPSFLSCSLQPMTRG

FIG. 10

RAT GLUTx nucleic acid sequence (1037)

TGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGGCAC
GAGCTGGTGCCCATCTCCGCAGAGCCTGCTGATGTTCACCTGGGGCTGGCCT
GGCTGGCTGTAGGCAGCATGTGCCTCTTCATCGCTGGTTTTGCAGTAGGCTG
GGGACCCATCCCCTGGCTCCTCATGTCAGAGATCTTCCCTCTGCACATCAAG
GGTGTGGCTACCGGCGTCTGTGTCCTCACCAACTGGTTCATGGCCTTTCTGG
TGACCAAAGAGTTTAACAGCATCATGGAGATCCTCAGACCCTACGGCGCCTT
CTGGCTCACCGCTGCCTTCTGTATCCTCAGCGTCCTTTTCACGCTCACCTTTG
TCCCTGAGACTAAAGGCAGGACTCTGGAACAAATCACAGCCCATTTGAGGGA
CGGTGACGGACCCCTTTCTGTGACTGGCAGCCCTGAGCTGAGCTGGCTTCGG
GTTTCAAAAGGAGTGGAGTGGCCTCAGTGACCACAGTTTGAGCCCAGGGGC
CCCCTGACTCCTCAGATTTCCGGGCCAGCTTTGTCCAGATCTCAACCCAGATT
CCACACCATGAGCTTCACCAGATTCTGAGGCTCNTGNAGCCTGCTGCACACA
CAGCACATTTGCGGGCTCCTGGCTCTAGTGCTCTGGCTGGGCATCTTTGGGG
TGCTTGGTCCTAAGCAACTGCCCATACCTCACTTGACTGGGGGATGAGAAAG
GGACTTAGCCACATAAGATTTGGGCTCAGAAACAAGGTCAGGTGAGTCCAG
GAAGAAAAGAGAATGGTTCTTGTCTTGTCAACCAAGTCCTTCTCAGAGTGCC
AAAGACCTCCGGATTCACCTTGGGGTTAGCCAGCTTACCCATCACTTACAGG
TTCTCTCCAACTCTCAGCTGGTCTCAGTGTCCTGGATCATTAGTCACCAGGTC
TTGTTGAGTTTCAGAAAAATAAAAGGCCTCTTTCCGTTCAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACTCGAGGGGGGGCC

FIG. 11

RAT GLUTx amino acid sequence (165)

WRPLZNZWIPRAAGIRHELVPISAEPADVHLGLAWLAVGSMCLFIAGFAVGWG
PIPWLLMSEIFPLHIKGVATGVCVLTNWFMAFLVTKEFNSIMEILRPYGAFWLT
AAFCILSVLFTLTFVPETKGRTLEQITAHLRDGDGPLSVTGSPELSWLRVSKGVE
WPQ

FIG. 12

MOUSE GLUTx nucleic acid sequence (282)

GAGCCTGCTGATGTTCACCTGGGGCTGGCCTGGCTGGCTGTAGGCAGCATGTGC
CTCTTCATCGCTGGTTTTGCAGTAGGCTGGGGACCCATCCCCTGGCTCCTCATGT
CAGAGATCTTCCCTCTGCACATCAAGGGTGTGGCTACCGGCGTCTGTGTCCTCAC
CAACTGGTTCATGGCCTTTCTGGTGACCAAAGAGTTTAACAGCATCATGGAGATC
CTCAGACCCTACGGCGCCTTCTGGCTCACCGCTGCCTTCTGTATCCTCAGCGTCC
TTTTCACG

FIG. 13

MOUSE GLUTx amino acid sequence (94)

EPADVHLGLAWLAVGSMCLFIAGFAVGWGPIPWLLMSEIFPLHIKGVATGVCVLTN
WFMAFLVTKEFNSIMEILRPYGAFWLTAAFCILSVLFT

FIG. 14

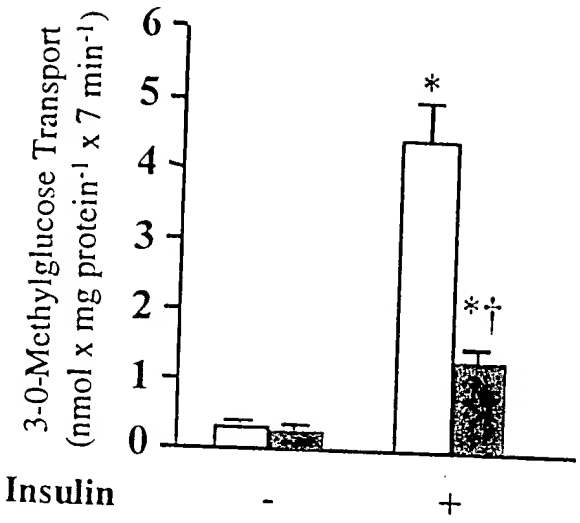
3-O-Methylglucose Transport
(nmol x mg protein-1 x 7 min-1)
6
5
4
3
2
1
0
*
*†
Insulin
-
+

FIG. 15

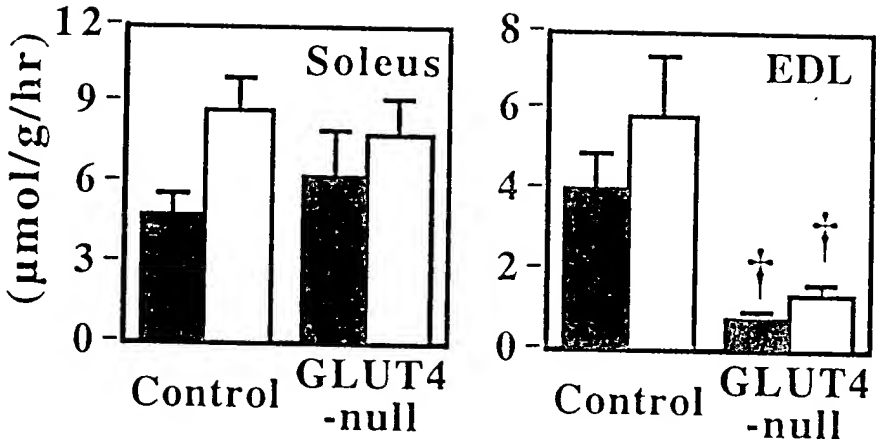
(μmol/g/hr)
12
9
6
3
0
Soleus
Control
GLUT4
-null
8
6
4
2
0
EDL
†
†
Control
GLUT4
-null

FIG. 16

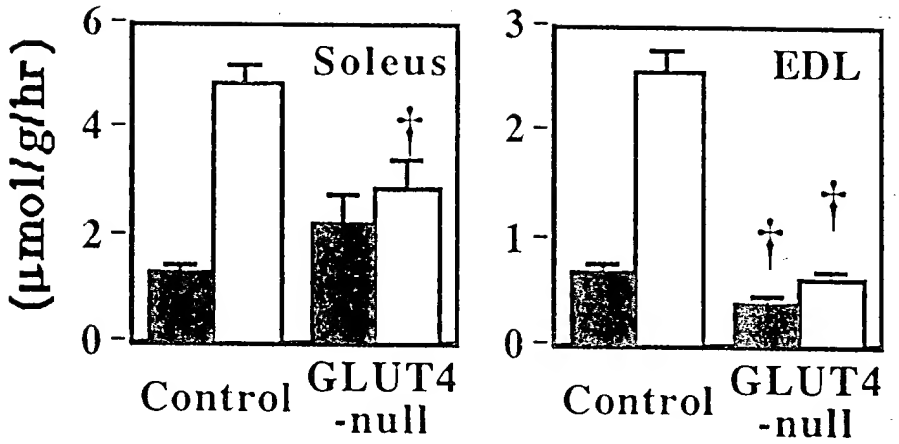
(μmol/g/hr)
6
4
2
0
Soleus
Control
GLUT4
-null
3
2
1
0
EDL
Control
GLUT4
-null

FIG. 17

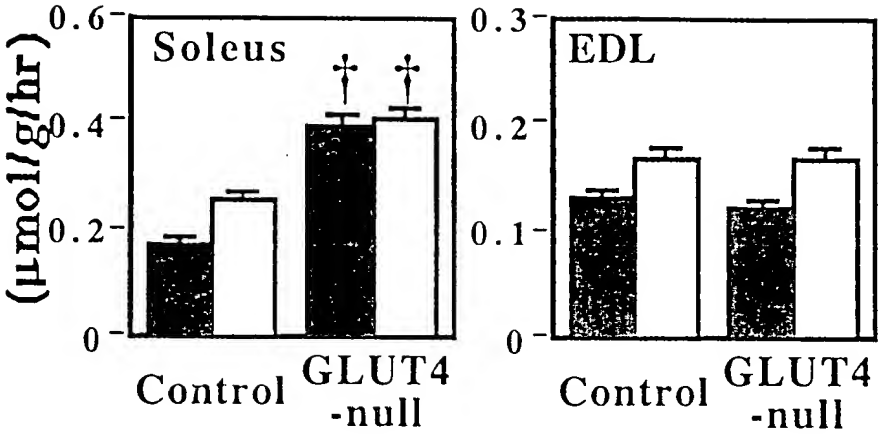
(μmol/g/hr)
0.6
0.4
0.2
0
Soleus
†
†
Control
GLUT4
-null
0.3
0.2
0.1
0
EDL
Control
GLUT4
-null

FIG. 18